

**ENHANCED ACTION OF SORGHUM AND COWPEA FLAVONOID  
MIXTURES AGAINST INFLAMMATION**

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2016

Major Subject: Food Science and Technology

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## ABSTRACT

Sorghum and cowpea contain structurally distinct flavonoids that may provide complementary bioactive properties. This study investigated the interactive effects of combining sorghum and cowpea flavonoids on inflammation and reactive oxygen species (ROS) generation in non-malignant colon myofibroblasts (CCD-18Co). The effect of sorghum-cowpea flavonoid combinations on ATP binding cassette (ABC) transporter proteins in Caco-2 cell model was also investigated.

The White sorghum-white cowpea extract combinations at (0.1 µg/mL) synergistically (1.5-2 times) attenuated LPS-induced ROS generation as well as LPS-induced inflammation in non-cancer CCD-18Co cells by inhibiting the activation of NF-κB and related downstream targets such as IL-6, IL-8 and TNF-α at both mRNA and protein levels. Combination of pure apigenin (dominant flavonoid in white sorghum) and quercetin (prevalent flavonoid in white cowpea) demonstrated similar enhanced action (1.5-2 times) in protecting the CCD-18Co cells against ROS generation and inflammation at 0.1 µM, indicating the flavones and flavonols in sorghum-cowpea mixtures may be responsible for the observed synergistic effect. The magnitude of synergy was strongly dependent on the relative ratios of sorghum-cowpea and apigenin-quercetin mixtures, which suggests different mechanisms of action for the flavones and flavonols.

The White sorghum-white cowpea as well as apigenin-quercetin combinations synergistically modified the activity of efflux transporters in Caco-2 cell monolayers by

inhibiting the expression of apically located ABC transporters including BCRP, MRP2, MRP3 and MDR1 at both mRNA and protein levels. These results suggest that synergistic down-regulation of efflux transporter proteins could be a mechanism for enhanced action sorghum-cowpea combination against inflammation.

Overall findings from these studies suggest that consuming sorghum and cowpea together may favorably influence health outcomes beyond what can be predicted from individual commodity. This is important because cereal and legumes are usually consumed together; strategic cereal and legume combinations might be formulated for optimized benefits in chronic disease prevention.

Further *in vivo* studies with sorghum-cowpea diets are required to assess the physiological effects of sorghum-cowpea flavonoids mixtures on biomarkers of inflammation and chronic disease.

## **DEDICATION**

### **TO MY BELOVED FAMILY**

My Supportive Husband Dr. Reza Ghasemi

as well as

My Splendid Mother Ms. Farhaneh Aleyassin, My respectable Father Mr. Ahmadreza  
Agah and My Lovely Sister Ms. Shabnam Agah

I am fortunate to have you all in my Life.



## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my committee chair, Dr. Joseph M. Awika, for this opportunity of my education, the consistent, dedicated but yet critical guidance and support throughout the course of this research. I would also like to thank my committee member, Dr. Susanne U. Talcott, for her selfless support and dedication, in terms of strengthening this research. I am grateful to have such splendid mentors. Grateful thanks are extended to my committee members, Dr. Bhimanagouda S. Patil and Dr. Peter S. Murano, for their thoughtful ideas and support during my study. I am indebted to all of them.

Grateful thanks goes to Dr. Hyemee Kim, for her excellent technical support in the cell culture studies, as well as the discussions to improve the research. Special thanks to Dr. Liyi Yang for her high quality technical assistance with the chemical analysis and data interpretation, as well as moral encouragement and inspiration during my study. Special thanks are also extended to Dr. William L. Rooney and Dr. B. B. Singh in the Department of Soil & Crop Sciences and Texas AgriLife Extension of Texas A&M University for providing sorghum and cowpea seeds used in this study.

I would to express my heart-felt thanks to all my colleagues and friends in both Cereal Quality Lab and the Talcott's Lab. I wish to thank Ms. Audrey Girard, Ms. Paula De Aguiar Cipriano, Mr. Derrick B. Amoaka, Mr. Vinicius Venancio, Ms. Julia Brantsen, Miss Shreeya Ravisankar, Mr. Tadesse F. Teferra, Ms. Sharris Vader, Miss

Lailah Abrao and Mr. Ryan Barnes for the tremendous assistance and support they provided for this research; without any of you, the research could not be as strong as it is. I would also like to thank Dr. Leonnard Ojwang, Dr. Tom Jondiko, Dr. Frederico Barros, Dr. Archana Gawde, Ms. Karla Siska, Dr. Victor Taleon, Ms. Amy Collison, Miss Dorothy Herrman, Miss Kristen Dunn and Mr. Taehoon Kim, for all the discussions we had which provoked my ideas and improved myself both in my expertise and my personality. I am very fortunate to have worked and spent the most important years of my higher education with all of you together. I shall appreciate all the moments we spent, either cheerful or difficult, which I carry as a fortune of my life.

Finally, I extend my everlasting gratitude to my family, my husband Dr. Reza Ghasemi, as well as my father Mr. Ahmadreza Agah, my mother Ms. Farhaneh Aleyassin and my sister Ms. Shabnam Agah, for their affection, encouragement, patience and faith in the career path I chose. I love you all.

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## CHAPTER I

### INTRODUCTION

Chronic inflammation plays an important role in a broad range of chronic diseases such as cardiovascular disease (CVD) and cancer (58, 67). Overproduced reactive oxygen species (ROS) can alter the redox status of cells and tissues, which can prompt activity of pro-inflammatory enzymes and result in modification in cell signaling pathways including inflammation (66, 73, 111, 131). In developed countries, CVD and cancer are the principal causes of death; and are expanding problems in developing countries (56, 118). Epidemiological evidence has documented a connection between consumption of whole grain and grain legume and lower risk of CVD, cancer, and diabetes, which may be associated with their dietary fiber and polyphenols among others (71, 79, 124). Cereals and legumes are traditionally consumed together to overcome protein deficiency, improve food security and provide required vitamin and minerals (44, 89, 137).

Sorghum is one of the prominent crops among the cereal grains, and has serious role in food security of semi and small-scale farmers in Africa (8, 28). Sorghum has high levels of polyphenols not commonly found in other cereal grains such as wheat, barley and oats (8, 31, 32). Cowpea is a highly nutritious, stress resilient legume that is a rich source of high quality proteins for many low-income populations (100). Sorghum and cowpea are both heat and drought tolerant and can be grown under environmental

stresses in places not suitable for the growth of other cereals and legumes, hence they are produced and consumed together (e.g. in west Africa) (6, 85).

Recent evidence demonstrates that sorghum and cowpea also contain distinctly different flavonoids with different structural profiles that may provide complementary bioactive properties (8, 92, 93). For instance, in sorghum, prevalent monomers including flavones, flavanones and 3-deoxyanthocyanidins are un-substituted at position 3 of the heterocyclic ring; whereas, the dominant monomeric flavonoids in cowpea are substituted at position 3 (glycosides of quercetin, anthocyanins and flavan-3-ols) (8, 92, 93). Additionally, the majority of monomeric sorghum flavonoids exist as aglycones, while large molecular weight flavan-3-ols (condensed tannins) are also present in sorghum. In cowpea, these compounds mostly exist as glycosylated monomers (8, 12, 92, 93).

The chemistry and bioactivity of flavonoids are highly influenced by their structural profiles. Literature demonstrates that flavonoids with different chemical structures block various inflammatory mediators by diverse cellular mechanisms (58, 70, 103, 129). Most of the flavonoids classes suppress the activity of nuclear factor- $\kappa$ B (NF- $\kappa$ B) (the main molecular link between inflammation and inflammatory-linked diseases) and mitogen activated protein kinase (MAPK), which are key regulators of inflammation. This leads to expression of several pro-inflammatory cytokines and enzymes associated with chronic inflammation disorders (67). Therefore, flavonoids present in plants that can effectively down-regulate NF- $\kappa$ B, pro-inflammatory cytokines and scavenge ROS may reduce inflammatory responses and alleviate the related pathological conditions.

Although the dietary ingestion of flavonoids and their metabolites's is high, their effective bioavailability (and by extension bioactivity) is low. This is partly due to their metabolism by phase II enzymes and/or increased metabolites transportation from intestinal cells back to the lumen (apical side), moderated by ATP binding cassette (ABC) transport proteins (18, 19, 76). Intestinal ABC transporters involved in flavonoid transportation, including breast cancer resistance protein (BCRP/ ABCG2), multidrug resistance protein (MRPs/ABCCs) and P-glycoprotein (*P*-gp/MDR1/ABCB1) are localized in the apical membrane (18, 19, 76). Several investigations have revealed that, flavonoids and their metabolites, based on their structure; might perform as inducers or inhibitors of ABC transporters (BCRP, *P*-gp and MRP2) (20, 34, 62, 82, 117). Most anti-inflammatory flavonoids including apigenin and quercetin and its glycosides generally act as inhibitors of transporter proteins (81, 120, 132). Therefore, flavonoids present in plants that can effectively down-regulate ABC transporters such as BCRP, MDR1, and MRP2 may enhance bioavailability and bioactivity of other flavonoids. Researchers have observed that health benefits and bioactive properties of food combinations cannot be explained by the properties of individual bioactive compounds alone. The ingestion of bioactive constituents are always in the form of natural combinations, hence, interactions among phytochemicals such as flavonoids contribute to the ability of natural foods to protect human health and mitigate disease damage. However, the investigations on phytochemical interactions are mostly restricted to *in vitro* antioxidant tests or purified polyphenol mixtures (2, 15, 52, 54, 60, 78, 98, 107, 109, 121, 135, 145). Few studies have been performed on combined benefits of natural

mixtures of flavonoid found in complementary foods often consumed together.

Sorghum and cowpea provide an intriguing opportunity to demonstrate the possible extent of enhanced benefits of consumption of cereal-legume mixtures; given the distinct differences in their flavonoid profiles.

Based on the evidences on relationship between flavonoids and transporter proteins (19, 24, 49, 101, 133), we hypothesized that the structurally different flavonoid classes in sorghum and cowpea will target different signaling mechanisms that will enhance their effect against markers of inflammation and oxidative stress. The overall goal of this study is to establish the interactive effect of the flavonoids present in sorghum and cowpea against markers of inflammation and ROS generation.

The goal is to understand how consuming foods together (in this case cereal and legumes) can favorably influence health outcomes beyond what can be observed by measuring individual components. This is especially important given that cereal and legumes are usually consumed together, and health benefits derived from such diets cannot be easily explained by laboratory studies based on individual components. This study will lead to optimized utility of strategic food combinations to prevent chronic disease.

Three research objectives were established to test our hypothesis

1. Determine interactive effects of sorghum and cowpea flavonoid mixtures against ROS generation in non-malignant colon myofibroblast CCD-18Co.
2. Determine interactive effects of sorghum and cowpea flavonoid mixtures against inflammation in non-malignant colon myofibroblast CCD-18Co.

3. Investigate the effect of sorghum-cowpea flavonoid mixtures on ATP binding cassette (ABC) transporter proteins using Caco-2 cell model.



## **CHAPTER II**

### **LITERATURE REVIEW**

#### **Whole Grains, Grain Legumes and Disease Prevention**

##### *Whole Grains, Grain Legumes and CVD and Cancer*

Chronic nutrition related disease such as cardiovascular disease and cancer are leading cause of death in the USA and in most industrialized countries. This trend is also growing in developing countries (71, 79, 124). Staple diet compositions are dependent on geographical or sociological factors, and have a marked impact on human health. Epidemiological studies have revealed that high dietary intake of products containing whole grain and grain legume is strongly associated with reduced risk of chronic diseases such as cardiovascular disease, cancer and diabetes (71, 79, 124).

Consumption of whole grains and grain legumes has been demonstrated to protect against various chronic disease. This is associated with the presence of dietary components such as dietary fiber, vegetable protein, resistant starch, oligosaccharides, polyphenols, phospholipids and fatty acids, phytosterols, as well as saponins (56, 59, 71, 75). For example, two recent studies demonstrated that high intake of whole grains revealed lower risk of total mortality (17%) and CVD mortality (9%) in US men and

women, independent of other dietary and life styles parameters (56, 139). Since similar inverse association was observed between bran intake (dietary fiber) and CVD mortality, the authors identify cereal fiber as the potential protective component in chronic disease prevention (56, 139). Other studies have found that the combination of whole grains' myriad bioactive constituents, rather than any one component such as cereal fiber, explains their protective effects in CVD (59).

Epidemiological evidence showed that high dietary intake of legumes (more than 4 times per week versus less than once a week) decreased the risk of coronary heart disease (CHD) and CVD by 22 and 11%, respectively (14). Consumption of common beans was associated with a 7% decrease in serum LDL-cholesterols and more than 10% decrease in triacylglycerols, with no significant alteration in serum HDL-cholesterol values (115). Common beans also contain high levels of folic acids and thiamine-vitamin, which are acknowledged to lower serum homocysteine concentrations (29). Essential minerals from legumes are known to reduce the risk for hypertension and stroke (7).

Several other epidemiologic studies showed inverse correlation between intake of whole grains and grain legumes and protecting against certain cancers. For example: In a recent prospective study, strong consistent inverse association was observed between high dietary intake of both whole grains and whole grain products and oesophageal cancer in Scandinavian countries (123). A large cohort study in the Netherlands, displayed strong link between French beans and broad beans with reduction in the risk of prostate cancer (118). A cohort study of non-Hispanic white California Seventh-day

Adventists in the United States revealed that individuals who consumed red meat with frequent intake of legumes had significant protection against colon and pancreatic cancers (39).

### **Importance of Cereal-Legume Combinations**

Cereals and legume are commonly important food crops with amino acid profiles that complement each other. For example, cereals are low in the amino acid lysine but adequate in the amino acid methionine. Legumes provide adequate lysine but are low in methionine. Cereal-legume combinations provide high-quality protein. Therefore, they are traditionally consumed together to overcome nutritional deficiency, improve food security, reduce hunger, and provide required vitamin and mineral to human body (44, 89, 137). The protein content of cereal grains including wheat, sorghum, oats and rice constitute 10 percent of their total calories; whereas the average of legumes protein content is about 20 to 30 percent of their calories (44, 89, 137).

Many cultures around the world rely on a cereal-legume combination to provide a major portion of their dietary energy including maize and beans in Latin America, millet and ground nuts in the African Sahel, sorghum and cowpea in west Africa, rice and soybeans in Southeast Asia, wheat and garbanzos in the Middle East, rice and dal in India, and corn bread and black-eyed peas in the southern United States (44, 89, 137).

### **Importance of Sorghum Bicolor (L.) Moench as a Cereal Grain**

Among the cereals, sorghum is the fifth most important crop and plays an important role as a food security crop and a source of income. Sorghum is the dietary staple food of more than 500 million people in more than 30 countries, especially in Africa, Asia, and the semi-arid tropics (8, 28, 125). The consumed forms of sorghum as human foods include couscous, porridge, baked goods (bread, flat bread, cookie, and steam bread), and fermented drinks in Africa, Asia and Middle East (8, 28). Sorghum has a good potential for gluten-free breads and other baked products like cakes and cookies, crackers, breakfast cereals and snacks (8, 28).

Among cereals, sorghum contains high levels of polyphenols compared to other crops. The main locations of polyphenols are the pericarp, testa, and aleurone layer in sorghum. In sorghum grains, genotypes can influence the composition of phenolic compounds (8, 11, 31, 33). Phenolic acids, flavonoids and proanthocyanidins are the polyphenolic groups present in sorghum (8, 11, 31, 33).

Evidence suggests an inverse correlation between intake of sorghum and protection against CVD and certain cancers. For example: A randomized crossover design showed that consumption of high dose (100 g) of sumac sorghum puff cereal daily (containing high levels of tannins and polyphenols) reduced the small dense LDL and increased the larger and less dense LDL; these are associated with reduced CVD risk profile (53). Another study showed that diets supplemented with sorghum kafirin extract (100 g (20%) sorghum flour) improved lipid metabolism and increased the serum antioxidant potential (67%) in rats (94).

Sorghum polyphenols have been associated with chemoprotective potential (142). For example: Flavones in sorghum induced apoptosis in nonmalignant colonocytes by the induction of estrogen receptor (143, 144). A recent study showed that Hwanggeumchal sorghum extracts (HSE) (40 µg/mL) inhibited the proliferation of human colon cancer cells in a dose-dependent manner by suppressing both Jak2/STAT3 and PI3K/AKT/mTOR pathways (26). Furthermore, HSE treatment suppressed tumor growth and pulmonary metastasis in animal models (26).

### **Importance of *Vigna Unguiculata* (Cowpea) as a Grain Legume**

Cowpea (*Vigna unguiculata*) is an annual African herbaceous legume, which is called “black-eyed beans”, “black-eyed peas” or “southern peas” in the United States; whereas in some parts of the world, it has various names such as *mkunde* (Swahili), *coupe* and *niebe* (French), *lobia* (India), *caupi* (Brazil), or *frijoles* (Spanish). In the semi-arid tropics covering Asia, Africa, southern Europe as well as Central and South America, cowpea is one of the most important food crops (35, 122). West Africa has the largest production and consumption of cowpea in the world (Nigeria and Niger). Among the total world production of 5.7 million tons, 2.5 million tons is produced in Nigeria (36).

Cowpea is relatively cost effective, nutritious and low glycemic index legume. Low GI foods have been shown to reduce post-prandial blood glucose and insulin responses in normal and diabetic patients (37, 100). Cowpea contains 20 to 30% proteins, which is rich in essential amino acids including lysine and tryptophan (37, 100).

Cowpea is extensively used in a vast array of food preparations, and is mainly consumed as a boiled, steamed, fermented or fried vegetable (128). In West Africa, the traditional commonplace foods utilizing recipes of cowpea are *ewa* (boiled whole bean), *akara* (deep-fried dehulled cowpea paste), *seke-sin* (fried cowpea cakes with green onions), *gbegiri* (cowpea soup) and *moin-moin* (steamed cakes) (1). Cowpea is consumed as cooked whole seed, or cooked green immature pods in the form of curry in conjunction with certain cereals in India (64). In southern US, it is canned and consumed as boiled beans (36).

A few studies have revealed that cowpea could have significant effects on reducing CVD and cancer due to the high levels of polyphenols and dietary fibers. A randomized cross over study showed that consumption of 25 g/day of cowpea protein isolate reduced total cholesterol (12%), LDL cholesterol (18.9%), non HDL cholesterol (16%) and increased HDL cholesterol (+2.7 %) in hamsters (42). In another study, the extracts of whole seed cowpea at a concentration of 100 mg (GAE/L) inhibited 65% the proliferation of hormone-dependent mammary (MCF-7) cancer cells (46). Ojwang *et al.*, (2012) revealed down-regulation of pro-inflammatory cytokines (IL-8, TNF- $\alpha$ , VCAM-1), transcription factor NF- $\kappa$ B and modulation of microRNA-126 (specific post-transcriptional regulator of VCAM-1) by four cowpea extracts (black, red, light brown, white) in dose dependent manner (2-20 mg GAE)/L) in LPS-induced non-malignant CCD-18Co cells (91).

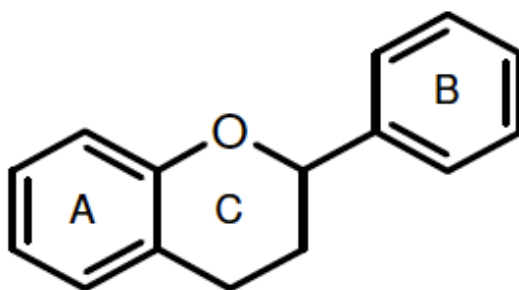
## **Importance of Sorghum-Cowpea Intercropping**

Sorghum and cowpea are both well adapted to semi arid regions and perform better than other cereals and legumes under environmental stresses (e.g. high heat and low moisture climate), thus they are produced and consumed together (e.g. in west Africa) (6, 85). When cowpea and sorghum are planted together, cowpea mainly relies on symbiotic nitrogen (N) fixation; while sorghum accepts more N from soil compared to the sole cultured cropping. This process is considered as complementary usage of N resources (6, 85). In consequence, sorghum-cowpea intercropping overcomes potential nutrient deficiencies and soil fertility. Musa *et al.*, (2012) reported that inoculation and intercropping of sorghum-cowpea improved the yield and quality of cowpea and contribute to food security, through the full exploitation of the land and rainwater potentials (85).

## **Flavonoids**

Flavonoids are pervasive and widely distributed in plants. Flavonoids are the most predominant group of polyphenols responsible for the pigmentation of flowers and leaves (67, 138). They are secondary metabolites derived from products of the Krebs cycle (acetyl Co A) and the aromatic amino acid biosynthesis (phenylalanine). The flavonoids general structure comprise a 15 carbon atom flavan nucleus organized in a C6-C3-C6 ring in which the two C6 units are of phenolic nature (67). Almost 5,000 flavonoids have been identified and classified into at least 10 chemical groups (138). Flavonols, flavanones, flavones, flavan-3-ols and anthocyanidins are the most prevalent

flavonoids identified and described, among the groups (67, 138). The hydroxylation pattern and variations in the chromane ring are the main differences between the chemical groups (67).



**Figure 1:** Basic diphenylpropane C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> skeleton.

### Flavonoids in Sorghum

The major subgroups of flavonoids that have been identified in sorghum grains are summarized in Table 1. In a diverse range of sorghums grains, 3- deoxyanthocyanidins, flavones and flavanones were the subgroups that were reported in high concentrations (31, 32). Sorghums are the only known edible source of the unique 3-deoxyanthocyanin pigments (34, 138). Lack of substitution at position 3 of the C ring in 3-deoxyanthocyanidins versus the anthocyanins (Figure 1), makes them more stable as pigments (11). The highest levels of 3-deoxyanthocyanidins are present in the red pericarp sorghum grains. The two groups of 3-deoxyanthocyanidins, which have been



identified in sorghum grains, are apigeninidin, luteolinidin and their derivatives (11). Luteolin and apigenin are the principal flavones in sorghum and have been reported in significant amounts in tan pericarp sorghum grains. Eriodictyol and naringenin are the dominant flavanones detected in sorghum (8, 31, 33). Most of the sorghum polyphenols are not found in other cereal grains in meaningful quantities (32).

**Table 1:** Flavonoids found in sorghum grains.

Flavonoids	Reference
<b>3-Deoxyanthocyanins:</b>	
Apigeninidin	(33, 43, 86)
Apigeninidin-5-glucoside	(87, 119)
Luteolinidin	(33, 43, 87)
5-Methoxyluteolinidin	(33, 119, 140)
7-Methoxyluteolinidin	(119)
5-Methoxyluteolinidin-7-glucoside	(140)
7-Methoxylapigeninidin	(33, 95, 140)
Luteolinidin-5-glucoside	(87, 119)
5-Methoxylapigeninidin	(119)
7-Methoxylapigeninidin-5-glucoside	(140)
<b>Flavan-4-ols:</b>	
Luteoforol	(13)
<b>Flavones:</b>	
Apigenin	(33, 45, 119)
Luteolin	(33)
7-O-Methyluteolin	(144)
7-O-Methylapigenin	(144)
<b>Flavanones:</b>	
Eriodictyol	(33)
Eriodictyol-5-glucoside	(45)
Naringenin	(33, 45)
Naringenin-7-o-glucoside	(144)
<b>Dihydroflavanols:</b>	
Taxifolin	(45)
Taxifolin-7-glucoside	(45)

## Flavonoids in Cowpea

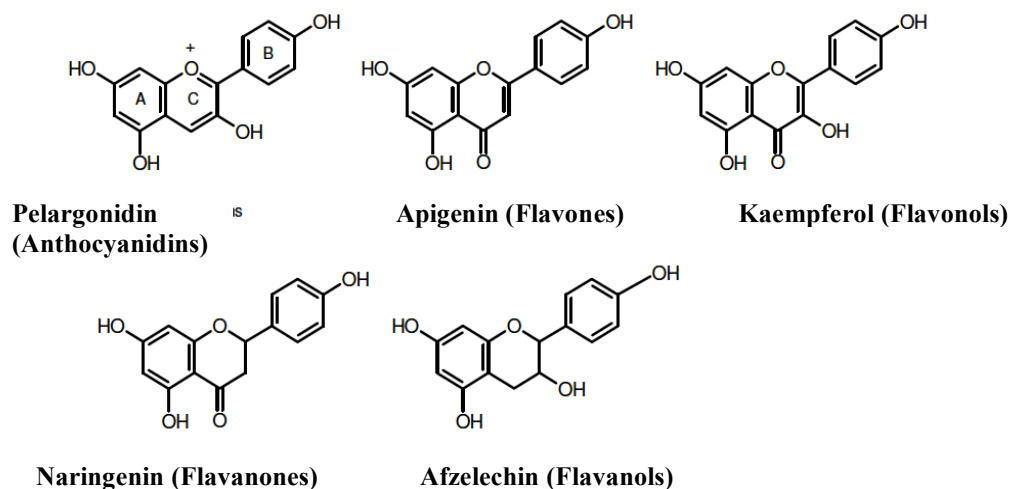
The major flavonoids have been identified in cowpea seeds are summarized in Table 2. In a diverse range of cowpeas seeds, flavonols, flavan-3-ols, anthocynins, proanthocyanidins are the subgroups that were reported in high concentrations (92, 93). Quercetin and its derivatives, followed by myricetin derivatives were the major flavonols constituents present in cowpea. Theses flavonols mainly exist in their glycosylated forms, often linked to glucose or rhamnose sugar moieties (92, 93, 134). Quercetin derivatives are found in most of cowpea varieties (92, 96). Ojwang *et al.*, (2012) identified acylated derivatives of flavonols in cowpea specifically malonoyl-, sinapoyl-, feruloyl-, acetoyl- and succinoyl- derivatives of quercetin glycosides. The authors demonstrated that flavonols are found in great amount in red cowpea phenotypes; whereas anthocyanins are found only in black and green cowpeas and the light brown cowpea had the highest flavan-3-ol content (92),

**Table 2:** Flavonoids found in cowpea seeds.

Flavonoids	Reference
<b><i>Anthocyanins:</i></b>	
Delphibidin-3- <i>O</i> -glucoside	(47, 51, 92)
Cyanidin-3- <i>O</i> -glucoside	(47, 51, 92)
Petunidin-3- <i>O</i> -glucoside	(47, 51, 92)
Malvidin-3- <i>O</i> -glucoside	(47, 51, 92)
Delphinidin-3- <i>O</i> -glucoside	(47, 92)
Cyanidin-3- <i>O</i> -galactoside	(47, 51, 92)
Peonidin-3- <i>O</i> -glucoside	(47, 51, 92)
Petunidin -5- galactoside	(92)
<b><i>Procyanidins:</i></b>	
Catechin	(93, 104, 134)
Catechin-3- <i>O</i> -glucoside	(93)
Epicatechin	(93)
Procyanidin-dimer	(93, 104)
Procyanidin-trimer	(93)
Procyanidin-tetramer	(93)
<b><i>Flavonols:</i></b>	
Quercetin- <i>O</i> -monoglucoside	(92, 134)
Quercetin- <i>O</i> -diglucoside	(92, 134)
Quercetin-3-(6"-malonoyl)-glucoside	(92)
Quercetin-3-feruloyl-diglucoside	(92)
Quercetin-3-di-acetoyl-diglucoside	(92)
Quercetin-3-(6"-sinapoyl)-rutinoside	(92)
Quercetin	(21, 80, 92)
Myricetin- <i>O</i> -monoglucoside	(21, 92)
Myricetin- <i>O</i> -diglucoside	(21, 92)
Kaempferol- <i>O</i> -diglucoside	(21, 92)
Quercetin-3- <i>O</i> -arabinosylglucoside	(92)
Quercetin-3- <i>O</i> -galactosylrhamnoside	(92)
Quercetin-3- <i>O</i> -glucosylrhamnoside	(92)
Quercetin-3- <i>O</i> -triglucoside	(92)
Myricetin- <i>O</i> -diglucoside	(92)

From table 1 and table 2, it is obvious that sorghum and cowpea contain structurally different flavonoids with little overlap in profiles (8, 92, 93). Thus, the mixtures of

sorghum-cowpea flavonoids may provide complementary bioactive properties.



**Figure 2:** Example of compounds in the major flavonoid classes.

### Importance of Flavonoids as Antioxidants

Oxidative stress is defined as an imbalance between productions of free radicals metabolites, (so called oxidants or reactive oxygen species (ROS)) and their elimination by protective mechanisms referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells (66, 109, 131).

ROS or free radicals include hydroxyl and peroxy radicals, hydrogen peroxide and singlet oxygen, among others. They are products of a normal cellular metabolism, and play vital roles in stimulation of signaling pathways in plant and animal cells (66, 73, 109, 131). Under sustained environmental stress, overproduced ROS impact

etiology of several chronic diseases such as chronic inflammation and cancer (66, 109, 131). ROS due to pro-oxidant activity are able to change the cellular and tissue redox status, which induces activity of kinases and phosphatases, and leads to alteration in cell signal transcription pathway for a variety of cellular response such as inflammation (66, 73).

Flavonoids and their glycosides, depending on their chemical structure (number and position of hydroxyl groups and conjugation and resonance effects), have varied antioxidant capacity through their ability to quench ROS and prevent oxidative damage (58, 69, 73, 131). Flavonoids can also act as antioxidants, through inhibition of enzymes, chelation of metal ions, reduction the free radicals formation, and regeneration of membrane-bound antioxidants (69, 74). Since structure facilitates electron delocalization across the molecule and contributes to an increase in hydrogen donation, chemical structure of flavonoids is the key factor that affects their radical scavenging capacity. For example: an *o*-diphenolic group (catechol-type structure) in B ring, a 2-3 double bond conjugated with the 4-oxo function, and hydroxyl groups in position 3 and 5 are structures that improve the flavonoid scavenging capacity (69, 74).

Quercetin, a flavonol that combines all of these characteristics, is one of the most potent natural antioxidants. The lack of one of these functionalities reduces the antioxidant ability (69, 74). For example: removal of one hydroxyl group from B ring twists the B ring 20° relative to A and C ring, which decreases electron delocalization and reduce the scavenging activity of kaempferol compared to quercetin (74). In the

same study, conjugation between the 3 hydroxyl group in C ring with the 4-oxo (carbonyl group) enhances delocalization electron from C ring in quercetin and made it a more potent radical scavenger versus luteolin (74). In taxifolin, the absence of 2,3 double bond reduces its antioxidant capacity versus quercetin (74).

Some studies show that biological interactions take place between flavonoids and other compounds both *in vitro* and *in vivo*. Frank *et al.*, (2006) showed that the inclusion of quercetin, catechin or epicatechin in the diet of rats protected the  $\alpha$ -tocopherol from oxidative degradation in blood plasma and liver tissue in the order of epicatechin> catechin>quercetin (38). Consistent with this animal study, the LDL isolated from plasma pre-incubated with the same flavonoids (50  $\mu$ M) was protected from free radical-induced oxidative damage resulting in high levels of  $\alpha$ -tocopherol (38).

Based on the structure function relationship between flavonoids and their antioxidant activity, we believe that the complementary effect of structurally different flavonoids in sorghum and cowpea may increase the health promoting effects of consuming specific sorghum-cowpea mixtures. An overview of main studies focused on assessment of flavonoid-flavonoid interactions on total antioxidant capacity are presented in the next part.

## **Importance of Flavonoids as Anti-Inflammatory Agents**

Inflammation is a natural response of the body to injury or infection caused by an internal or external factor such as bacterial, viral, parasitic and chemical agents (58, 67). The main function of inflammation is to resolve the infection or repair the damage and return to a state of homeostasis. The ideal inflammation response is rapid and destructive, yet specific and self-limiting. Chronic inflammation is the result of an imbalance between the inflammation trigger and inflammation response, which cause more damage to the host than the trigger (58, 67). Thus, when this process turns uncontrolled it may lead to several chronic diseases including cancer.

Several studies have shown that inflammation produces ROS, which leads to oxidative damage and increase the risk of DNA mutations and many further leads to cancer. In general, cytokines are a group of substances produced by resident or migrating cells (e.g. mast cells, macrophages and neutrophils), and function at many steps of the inflammatory response to heal wounds and stimulate epithelial cell proliferation (58, 67, 103). However, if these are uncontrolled they could lead to dysplasia and ultimately cancer. For example: Pro-inflammatory cytokines such as interleukins (IL-6) and tumor necrosis factor (TNF- $\alpha$ ) are substances produced by immune cells (e.g. mast cells, macrophages and neutrophils). TNF- $\alpha$  is considered as a master switch for inflammation to cancer and is also known to activate NF- $\kappa$ B in immune system (90). IL-8 promotes phagocytosis of neutrophils, an event that causes secretion of ROS and has been implicated in the etiology of several chronic disorders such as colon cancer (90).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor that plays the key roles in regulating inflammation. NF- $\kappa$ B induces the expression of several pro-inflammatory cytokines genes, which are associated in carcinogenesis development and other chronic inflammation disorders (90, 108, 129). Thus, NF- $\kappa$ B plays a major role in the immune, stress, proliferative, apoptotic and inflammatory response. NF- $\kappa$ B is sequestered in the cytoplasm, where it is held inactive in a non-DNA-binding form by the inhibitory proteins (I $\kappa$ Bs) (90, 108, 129). When cells are stimulated with various NF- $\kappa$ B inducers, I $\kappa$ B proteins become rapidly phosphorylated by I $\kappa$ B kinase (IKK) complex – an inhibitor of NF- $\kappa$ B kinase. Thus, inhibition of NF- $\kappa$ B is regarded as a useful strategy for treatment of inflammatory disorders (90, 108, 129).

Flavonoids depending on their chemical structure suppress several inflammatory mediators via diverse cellular mechanisms (67, 68, 103). For example: Flavonols (quercetin, kaempferol) were found to be phospholipase A2 (PLA2) and lipoxygenase (LOX) inhibitor, but flavone class (apigenin) did not show any inhibitory effect (67, 103). In contrast, flavones (apigenin, luteolin) showed inhibitory effect on cyclooxygenase (COX) (67, 103, 108). Apigenin inhibited LPS-induced IL-1 $\beta$  production by inhibiting caspase-1 activation and also prevented LPS-induced IL-6 production by reducing the mRNA stability via inhibiting ERK1/2 activation (149). In another study, apigenin (10  $\mu$ M) suppressed LPS induced COX-2 expression in RAW 264.7 cells by inhibiting arachidonic acid (AA) release causing suppression of PGs synthesis (72).



The majority of flavonoids classes suppress the activity of nuclear factor- $\kappa$ B (NF- $\kappa$ B) through blocking of phosphorylation and degradation of I $\kappa$ B $\alpha$  by inhibiting IKK activation and suppression of the phosphorylation and nuclear translocation of p65. Quercetin was shown to down-regulate the activation of NF- $\kappa$ B pathway in a dose dependent manner by inhibiting the phosphorylation of I $\kappa$ B- $\alpha$  protein induced by LPS in macrophages, which resulted in reduction in TNF- $\alpha$  and IL-1 $\beta$  secretion by almost 40% (23). Quercetin was also reported to reduce the gene expression of specific factors implicated in local vascular inflammation including IL-1R, Ccl8, IKK, and STAT3 (68). A growing number of *in vitro* and *in vivo* studies indicate that combinations of dietary anti-inflammatory compounds can often results in significant activities at concentrations where any single agent is inactive. An overview of main researches focused on interactive effects of phytochemical mixtures against inflammation is presented in next part.

### **Synergistic Interaction of Phytochemicals**

Structurally diverse flavonoids may possess similar, overlapping, or different but complementary effects in their bioactive properties (54, 135). A combination of different plant-based foods may exhibit additive, synergistic, or antagonistic interactions among their different phytochemicals. Synergism between phytochemicals may arise via different mechanisms (2, 69). Antioxidant network in the membranal systems is an example of regeneration mechanism in which vitamin C regenerates vitamin E from its oxidized form, thus restore its original function (2, 69). Another synergistic

mechanism is the sacrificial oxidation mechanisms in which, several green tea polyphenols (epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (EGC), and gallic acid) reduce  $\alpha$ -tocopheroxyl radicals to regenerate  $\alpha$ -tocopherol. In addition, the green tea polyphenols were able to trap the initiating and propagating radicals, which prevent lipid peroxidation (2, 69).

Several studies have shown that plant flavonoids synergistically interact to enhance bioactive properties. For example: A combination of wild bilberry, cranberry, elderberry, raspberry and strawberry exhibited higher antioxidant capacity compared to the individual berries (135). The same study revealed that combining specific foods across categories was more (2 times) likely to result in synergistic antioxidant capacity than combinations within a food group. For instance: Among all the food combinations patterns tested, combining raspberry and adzuki bean extracts displayed highest synergistic interactions in four chemical-based assays including ORAC, DPPH, TPC and FRAP (135). In another study the combined extracts of oak (200  $\mu\text{g/mL}$ ) and rosemary (150  $\mu\text{g/mL}$ ) showed higher antioxidant activity (2 fold) than the individual extracts (107).

Sorghum-cowpea composite porridge revealed higher radical scavenging activity than the individual composite porridges by almost two fold, which indicates that sorghum containing flavones, flavanones and flavan-3-ols as prominent flavonoids and cowpea with flavonols and flavan-3-ols as dominant flavonoids can complement each other (6). The combination of natural antioxidant isolated from spinach leaves (2.4  $\text{mg/mL}$ ) with

50 $\mu$ M of ferullic acid, caffeic acid and EGCG were more effective (2.2, 3, and 2.6 fold, respectively) in scavenging ROS production versus the additive effect of individuals (109).

Until now few studies have focused on the assessment of phytochemical interactions in cellular pathways such as inflammation. Phytochemicals could interact by blocking one or more targets of the signal transduction pathways, increasing the bioavailability of the other phytochemical, or by stabilizing one another in the system (22, 48, 50, 116). Literature showed that the mixtures of foods containing different flavonoids were able to modulate genes, which were not significantly modulated by individual foods (69).

Park *et al.*, (2011) reported that co-treatment of luteolin and chironic acid more potently inhibits TNF- $\alpha$  and IL-1 $\beta$  (50%) as well as I $\kappa$ B $\alpha$  (70%) and p65 (60%) protein expression than the individual treatments at the minimum effective dose tested (12  $\mu$ M) in LPS induced RAW 264.7 cells; which resulted in synergistic down regulation of NF- $\kappa$ B activation (97). Hazewindus *et al.*, (2012) reported that in lycopene, ascorbic acid and  $\alpha$ -tocopherol combinations, lycopene mitigated inflammation response; whereas ascorbic acid and  $\alpha$ -tocopherol efficiently inhibited lipid peroxidation. Both activities complemented each other, which resulted in synergistic reduction of inflammation process (50). An animal study by Wu *et al.*, (2015) demonstrated that the mixtures of  $\beta$ -carotene (10 mg/kg) with low doses (50 mg/kg) and high doses (100 mg/kg) of quercetin synergistically increased the ratio of cytoplasm/ nucleus NF- $\kappa$ B protein levels by almost 50% as well as the levels of TNF- $\alpha$  and IL-6 in plasma compared to the individual treatments (140). Therefore, cooperation among functional compounds better explains

the health benefits of whole foods, than pure compounds tested in isolates.

### **Flavonoids as ABC Transporter Proteins Modulators**

ATP binding-cassette (ABC) transporter proteins are active trans-membrane proteins that utilize energy of adenosine triphosphate (ATP) to carry out certain biological processes including translocation of various substrates across membranes. ABC transporters are classified based on the sequence and organization of their ATP binding cassette domain (18, 19, 82). They use the energy to drive conformational change in trans-membrane domain. The relative binding affinities between the substrates and ABC transporter conformations determine the net direction of transport (18, 19, 82). ABC transporters are specifically located in apical (lumen side) or basolateral (blood/ plasma) side membrane of enterocytes (18, 19). In the intestine, ABC transporters located in apical side can limit the oral bioavailability of their substrates by mediating direct intestinal excretion back to the lumen; while the transporters located in basolateral side facilitate their substrate uptake into the blood (18, 19).

As previously discussed, flavonoids have various biological activities, such as anti-inflammatory, anticancer and antioxidant activity, which could affect several signaling pathways and enzymes that are involved in chronic diseases (3, 61). In order to exert these biological properties, flavonoids must be absorbed across the gut wall (3). In general, the absorption of flavonoids is a rapid process. Flavonoids, after entering the enterocytes, are subjected to extensive metabolism by intestine conjugating enzymes. Flavonoids undergo glucuronidation by uridine-5-diphosphate glucuronosyltransferase

(UGTs) and/or sulfation by sulfotransferase. These glucuronides and sulfates of flavonoids could be subsequently excreted into the lumen or bile by ABC transporters (3, 61). Thus, it is not surprising that despite high dietary intake of flavonoids and their metabolites, their effective bioavailability (by extension bioactivity) is limited (18, 19, 82). This can be related to the fact that significant metabolism of dietary flavonoids by phase II enzymes occurs in intestinal epithelial cells increasing efflux of their conjugated metabolites into the apical sides of epithelial cells by ABC transporters (18, 19, 82).

Intestinal ABC transporters, which are involved in flavonoid transport, including breast cancer resistance protein (BCRP/ ABCG2), multidrug resistance protein (MPPs/ABCCs) and P-glycoprotein (*P*-gp/MDR1/ABCB1) are localized in the apical membrane (18, 19).

Evidences demonstrates that flavonoids are not only substrates of active efflux transporters, but also, depending on their chemical structures, are well-known inducers and inhibitors of ABC transporters such as BCRP, MRP2 and *P*-gp, which are present in epithelial cells throughout the intestinal tract (3, 61). Various mechanisms have been reported for flavonoids-ABC transporter interactions. For example: flavonoids such as quercetin and genistein have been shown to inhibit the ATPase activity of ABC transporters (3). Another possible mechanisms for flavonoid inhibitory effect against transporters is via competitive inhibition; when flavonoids act as substrates which bind to the binding site of transporter (3). Furthermore, flavonoids can also modify the expression of ABC transporters at both mRNA and protein levels. This scenario is even more complicated due to the presence of multiple binding sites providing the conditions

for different kind of interactions (3, 61). Major interactions between flavonoids with key transporter proteins are discussed below:

### **Flavonoids as P-glycoprotein (*P*-gp) Modulators**

P-glycoprotein (*P*-gp), encoded by the ABCB1 gene, is the first human ABC transporter that was cloned and characterized through its ability to confer a multi-drug resistant phenotype to cancer cells. *P*-gp is mainly responsible for outward transportation (i.e., efflux) of hydrophobic, cationic or neutral compounds (61). Among the P-glycoprotein (*P*-gp) group, multi-drug resistant protein1 (MDR1) showed the highest expression level in human intestinal epithelial Caco-2 cell monolayers (61). Flavonoid aglycones such as quercetin, kaempferol hesperitin and naringenin exhibited inhibitory effect on *P*-gp by increasing vincristine uptake in MBEC4 cells (10-50  $\mu$ M). However, their corresponding glycosides were reported to have little or no effect on *P*-gp (80). There are at least two binding sites on *P*-gp with different binding properties for flavonoids. Binding to one of these sites has been shown to influence binding to the other site and the transport activity (3, 61, 81). Shape parameter and hydrophobicity are two major physico-chemical factors responsible for the affinity of flavonoid derivatives towards *P*-gp sites (3, 61, 81). For example: the flavonol group (quercetin and kaempferol) depending on their concentrations has a biphasic effect on vincristine (a chemotherapy medication used to treat a number of cancer type) efflux, causing inhibition or enhancement (3). The *P*-gp functionality changed at different concentrations. At a low concentration (10  $\mu$ M), *P*-gp phosphorylation was enhanced

which resulted in its higher activity; whereas at high concentration (50  $\mu M$ ), the activity of *P*-gp was hindered (81). Other related studies also revealed that flavonoids have an inhibitory role on *P*-gp in several cell lines such Caco-2 cells based on their ability increase cellular uptakes of various substrates (3). We are not aware of any studies that have determined the interactive effect of flavonoid combinations on *P*-gp protein modulation.

### **Flavonoids as Multidrug Resistant Protein (MRPs) Modulators**

MRPs is an efflux transporter, which belongs to the ABCC (also called MRP) subfamily of ABC transporters (61). The main function of MRPs, which is located to apical membrane of polarized cells, is to actively transport endogenous (e.g. bilirubin and its polar conjugates) and xenobiotic (e.g. drugs) substances out of the cells (61). Among the multidrug resistance proteins, MRP2 followed by MRP3 showed the most abundant expression levels in human intestinal epithelial Caco-2 cell monolayers (61). A study showed that chrysin, a flavone, play a dual role in regulating MRP2 in Caco-2 cells, which showed both inhibition and enhancement in expression of MRP2 (117, 120).

Quercetin inhibited MRP2 in a dose dependent manner (132). The same study showed that major phase II quercetin metabolites (glucoronides of quercetin) inhibited MRP2 with a similar potency to quercetin and also significantly increased the potential of quercetin to inhibit MRP2 in Sf9 inside-out vesicles (recombinant membrane vesicles that are robust and predictive model for studying transporter mediated drug interactions) (132).

Among 29 structurally different tested flavonoids, myricetin and robinetin were the two best MRP2 inhibitors in MDCKII cells at 15 and 22  $\mu\text{M}$  concentrations respectively, by inhibiting calcein efflux, indicating that a flavonol B-ring pyrogallol group appeared to be an essential structure for inhibition of MRP2 protein (133).

The only research that determine the effect of phytochemicals combinations on MRPs proteins found that the co-incubation of quercetin, chrysin, genistein and resveratrol in equal concentrations and ratios (50  $\mu\text{M}$ ) increased ochratoxin A (a food-borne mycotoxin) cellular accumulation and transportation to the basolateral (blood) side in Caco-2 cells by almost 50% and 80% respectively (3). It was hypothesized that more than one interaction might take place simultaneously; positive cooperation between substrates as well as between substrates and non-substrates. Polyphenols may exert this effect through competitive inhibition of MRP2 (3).

### **Flavonoids as Breast Cancer Resistant Protein (BCRP) Modulators**

Breast cancer resistance protein (BCRP), which is encoded by the ABCG2 gene, has an important role in limiting oral bioavailability of drugs and drug transport across the blood– brain barrier, blood–testis barrier and the maternal–fetal barrier of selected substrates (61). BCRP is broadly distributed in humans including kidney, liver and intestine (61). The substrates of BCRP include various compounds from endogenous to exogenous, which are generally organic anion or neutral substances (61).

The inhibitory effect of several flavonoids including apigenin, quercetin, kaempferol, naringenin and hesperitin against BCRP by increasing mitoxantrone accumulation has



been reported (57, 128,148). A few studies have revealed synergistic inhibitory effect on BCRP by multiple flavonoid mixtures (4, 18,147). Apigenin, bichanin A and chrysin combinations in equal molar concentrations exhibited synergistic inhibition on BCRP (147). Brand *et al.*, (2008) demonstrated that co-incubation of hesperetin with quercetin significantly reduced the apical efflux and improve it's excretion on basolateral side of Caco-2 cells, representing enhanced bioavailability (18). Another study demonstrated that multiple flavonoid combinations including Kaempferide, biochanin A, 5,7-methylflavone and 8-methylflavone in equal molar concentrations (2.5-5  $\mu M$ ) exhibited stronger BCRP inhibition (10 fold) versus the individual flavonoids, which lead to increasing mitoxantrone accumulation in breast cancer cells (4).

Structure-inhibition relationship has been established between flavonoids and ABC transporters. It appeared that a hydroxyl group at position 5, double bond between position 2 and 3 and a methoxyl moiety at position 3 or 6 are essential structures for BCRP inhibition (101, 135).

# **CHAPTER III**

## **INTERACTIVE EFFECTS OF SORGHUM AND COWPEA FLAVONOID MIXTURES AGAINST ROS GENERATION IN NON-MALIGNANT COLON MYOFIBROBLAST CCD-18CO CELLS**

### **Introduction**

Overproduction of reactive oxygen species (ROS), can change the cellular and tissue redox status. This process would induce activity of pro-inflammatory enzymes and lead to alteration in cell signaling pathways (52, 111, 131). Hence, overproduced ROS impact etiology of several chronic diseases such as chronic inflammation, CVD and cancer (111, 131). Cardiovascular disease (CVD) and cancer are the leading causes of death in developed countries; and are growing problems in developing countries (71, 124). Diet has a marked impact on incidences of chronic disease. For example, a link between whole grains and grain legumes intake and reduced risk of cancer, CVD, and diabetes has been observed in several epidemiological studies (71, 79, 124).

Cereals and legumes are traditionally consumed together to overcome protein deficiency, improve food security and provide required vitamin and minerals (44, 89, 137). Among the cereal grains, sorghum is one of the major crops and plays an important role in food security of semi arid small-scale farmers as well as a source of income in Africa (8, 31, 33). Sorghum has been recently shown to contain high levels of polyphenols compared to other crops (8, 28). Cowpea is a stress resilient legume that provides high quality proteins to many low-income populations (100). Cowpea has good

quality proteins, carbohydrates, polyphenols (100). Sorghum and cowpea are both well adapted to semi arid regions and perform better than other cereals and legumes under environmental stresses (e.g. high heat and low moisture climate), thus they are produced and consumed together (e.g. in west Africa) (6, 85). Recent evidences demonstrate that sorghum and cowpea also contain distinctly different flavonoids with different structural profiles that may provide complementary bioactive properties (8, 91, 92, 93). The majority of monomeric sorghum flavonoids lacks a hydroxyl (OH) group at position 3 of heterocyclic ring (flavones, flavanones and 3-deoxyanthocyanidins) and exists mainly as aglycones; whereas the dominant monomeric cowpea flavonoids (glycosides of quercetin, anthocyanins and flavan-3-ols) are substituted at position 3 and are mostly glycosylated (8, 31, 33, 92, 93).

Flavonoids, depending on their chemical structures have varied antioxidant capacity (27, 58, 69, 73, 131). Synergism in structurally different antioxidants may arise via diverse mechanisms including regeneration and sacrificial oxidation mechanisms. Researchers have recognized that the health benefits of food mixtures cannot be explained by the action of individual bioactive compounds in isolation. Responsible bioactive constituents such as flavonoids are always ingested in the form of natural combinations and never work independently. The beneficial effects of theses natural combinations are based on complex interactions. Consequently, the ability of natural foods to protect human health and mitigate disease damage is difficult to assess based on isolated compounds.

However, the majority of investigations on phytochemical interactions are still limited to tests of different compounds in a specific food and the purified phytochemical mixtures (2, 52, 107, 109, 121, 135, 145). Few studies have evaluated the complementary effect of structurally different flavonoids on key markers of disease prevention. Sorghum and cowpea provide an intriguing opportunity to demonstrate the possible extent of enhanced benefits of consuming cereal-legume mixtures. Here, we test the interactive effect of the structurally distinct sorghum and cowpea flavonoids on ROS quenching.

## **Materials and Methods**

### *Plant Materials*

Four cowpea varieties, including: IT98K-1092-1 (black), IT97K-1042-3 (red), 09FCV-E CB27 (white) and 09FCV-CC-27M (brown) were gathered in experimental station in College Station, Texas in July 2011. Three sorghum varieties, including: ATX635×RTX436 (white), TX430 (black), and High Tannin were provided by Dr W. L. Rooney in Department of Soil and Crop Sciences, Texas AandM University. The sorghum grains were harvested in 2011 at College station, TX and stored in -20 °C until use. Sorghum and cowpea varieties selection was based on their distinct differences in phenolic profiles basis (Table 3 and 4).

**Table 3:** Characteristics of sorghum varieties used in this study.

Variety	Pericarp Color	Secondary Plant Color	Major Compounds
ATX635×RTX436	White	Tan	Phenolic-acids, Apigenin
TX430 (black)	Black	Purple	3-Deoxyanthocyanins, Phenolic-acids, Flavones (apigenin, luteolin)
High Tannin	Red	Red	Proanthocyanidins

**Table 4:** Characteristics of cowpea varieties used in this study.

Variety	Pericarp Color	Secondary Plant Color	Major Compounds
09FCV-E CB27	White	White, black-eyes	Flavonols (quercetin derivatives)
IT98K-1092-1	Black	Black, white-eyes	Anthocyanins, Flavonols
IT97K-1042-3	Red	Red, white-eyes	Flavonols, Flavan-3-ols
09FCV-CC-27M	Brown	Light-brown, white-eyes	Flavonols, Flavan-3-ols

### *Chemicals and Reagents*

Pure standards used in this investigation were as follows: Apigenin was from Indofine (Indofine Chemical Company, Inc., Hillsborough, NJ); Quercetin was from Sigma-Aldrich Chemicals, St. Louis, MO. Folin-Ciocalteu reagent, ethanolamine, gallic

acid monohydrate, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, sodium fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), 2',7'-dichlorofluorescein diacetate (DCFH) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) was obtained from Wako Chemicals USA (Richmond, VA). All other chemicals were analytical grade.

#### *Sample Preparation*

Whole kernel sorghums and dry seed cowpeas were ground in a cyclone mill (UDY, Boulder, CO) to pass through 0.1 mm screen before extraction. Grounded samples were extracted with 70% (v/v) aqueous acetone with stirring for 2 h. Supernatant was collected by centrifuging ( $3100 \times g$ ) for 15 min at 4 °C and were used for antioxidant capacity assay. For cell cultural studies, acetone was immediately removed from the supernatant under vacuum at 40 °C by a rotary evaporator (Rotovap, Büchi, Flawil, Switzerland). The aqueous phase was freeze-dried and used as a crude extract. Extracts were kept at -80 °C until use. Freeze-dried white sorghum and white cowpea extracts were reconstituted at 10 mg/mL with 50% aqueous methanol (HPLC grade, EMD Millipore, Billerica, MA) for UPLC-MS identification.

### *Determination of Total Phenols Content (TPC)*

The total phenols content (TPC) were determined by the Folin-Ciocalteu assay described by Kaluza *et al.*, 1980 using gallic acid (GA) as the standard (65). The ground sorghum and cowpea samples (0.1 g) were separately extracted with 70% aqueous acetone (10 mL) by continuously shaking for 2 h on a Standard Analog Shaker (VWR, USA) at ambient temperature, centrifuged (10000 g-force for 10 min) and the supernatants gathered. For each individual sorghum and cowpea variety, the extractions were conducted in triplicates.

Distilled water (1.1 mL) were added into the extracted aqueous samples (0.1 mL) then reacted with 0.4 mL Folin reagent and 0.9 mL 0.5 M ethanolamine for 20 min at room temperature. The absorbance was measured using a UV-visible spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 600 nm against a reagent blank. The TPC values were expressed as micrograms of gallic acid equivalent per gram of sorghums and cowpeas ( $\mu\text{g GAE/g}$ ) on dry weight basis through the calibration curve of gallic acid.

### *Identification of Compounds by UPLC-ESI-MS Analysis*

The sample aliquots were analyzed on a Waters - ACQUITY UPLC/MS system (Waters Corp., Milford, MA), which was equipped with a column heater, sample manager (autosampler), binary solvent manager; and Photodiode array  $e\lambda$  (PDA) and interfaced with an electro spray ionization (ESI) source Mass Spectrometer equipped with a tandem quadrupole (TQD) detector. Flavonols were monitored at 360 nm; while flavones monitoring wavelength were 340 nm. A Kinetex C18 column, (100  $\times$  2.10 mm,

2.6  $\mu\text{m}$  (Phenomenex, Torrance, CA) was used for separation at 40°C with 0.4 ml/min flow rate. The 0.05% formic acid in H<sub>2</sub>O (solvent A) and acetonitrile (solvent B) were used with the following gradient: 5% B from 0 – 2 min, 5 – 75% B from 2 – 27 min, 75 % B isocratic from 27 – 30 min, 75 – 5 % B from 30 – 31 min, followed by 5% B isocratic for 5 min to allow for column equilibration before the next injection. Mass spectrometric data was acquired in negative mode for flavonols and their derivatives as well as flavones. The aliquots injection volume was 1  $\mu\text{L}$ . Data were analyzed and acquired by *Empower 2* software (Waters Corp., Milford, MA). For both flavones and flavonols, the MS scan was recorded in the range of 100-1500 Da. Argon was used as the collision gas, while nitrogen was used both as a drying gas and as nebulizing gas. The gas flow conditions for nitrogen were 800 and 50 L/h for desolvation and at the cone, respectively. Source block temperature and desolvation temperature were set at 150 and 400°C, respectively. Optimization of ionization conditions was based on the intensity of the mass signals of protonated/deprotonated molecules and aglycones fragments, and was performed for each individual peak/compound detected. Mass parameters were optimized as follows: capillary voltage, 3.0 kV; and cone voltage, 30 V for negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage, 30-55 V; and collision energy, 15-40 V, respectively. Identification of compounds was performed by corresponding commercial standard UPLC retention times, UV-Vis spectra and MS data with authentic standards. In cases where standards were not available, identification were performed based on comparing fragment elution profile, and UV-spectra to publish the data.



#### *Trolox Equivalent Antioxidant Capacity (TEAC) Assay*

The end point based TEAC assay as described by Awika et al 2003 was used to measure the ABTS scavenging activity of sorghum and cowpea extracts. Three replicates of the ground sorghum and cowpea samples (0.1g) were separately extracted with 70% aqueous acetone (10 mL) by continuously shaking on a Standard Analog Shaker (VWR, USA) at room temperature for 4 h and centrifuged (10000 g-force for 10 min). The supernatants were used to determine the scavenging activity of ABTS<sup>+</sup>. A mixture of ABTS (8 mM) and potassium persulfate (3 mM) in distilled water was prepared and reacted at room temperature for 12 h in the dark. The ABTS working solution was prepared prior to the assay by diluting 10 mL ABTS stock solution with 290 mL of pH 7.4 PBS buffer to an absorbance of 1.5 at 734 nm. The aqueous sample solutions (0.1 mL) was added to the diluted ABTS working solution (2.9 mL) and reacted at ambient temperature for 30 min. These were performed in triplicates. The radical scavenging activity was expressed as  $\mu\text{mol Trolox equivalent/g sample on dry weight basis}$  [ $\mu\text{molTE/g (db)}$ ]. Trolox was used as a standard and results ( $\mu\text{mol TE/g}$ ), dry weight basis (9).

#### *Oxygen Radical Absorbance Capacity (ORAC) Assay*

The kinetic based oxygen radical absorbance capacity (ORAC) method developed by Cao et al, 1993 was used to measure the antioxidant activity of sorghum and cowpea extracts according to the method described by Awika et al 2009 using a Biotek Synergy HT plate reader with automatic dispenser (Biotek, Winooski, VT).

The ground sorghum and cowpea samples (100 mg) were separately extracted in 70% aqueous acetone (10 mL) in triplicates for 1 h at 4°C and then shaken for 1 h at ambient temperature. After centrifuging, 4 mL aliquots of the supernatants were transferred into clean test tubes and stored at 4°C until analyzed. Prior to the analysis, the extracts were properly diluted with distilled water to fit within the linearity range of the Trolox standards. The diluted aqueous sorghum and cowpea extracts (25  $\mu$ L) and Trolox standards (25  $\mu$ L) were analyzed on a solid black 96-well plate in triplicates. The Trolox standards (0, 6.25, 12.5, 25, 50 and 100  $\mu$ M) and sodium fluorescein working solution ( $4 \times 10^{-3}$   $\mu$ M) and 2,2'-azobis-2-methyl-propanimidamide dihydrochloride AAPH (153 mM) were prepared using PBS buffer (pH 7.4). Prior to incubation, 150  $\mu$ L of sodium fluorescein working solution was added to each well and the microplate (sealed with film) was incubated for 30 min in the plate reader set at 37°C. After the incubation period, 25  $\mu$ L of the peroxy generator (153 mM AAPH) was automatically dispensed into appointed wells according to the layout to initiate the oxidation reaction.

After shaking the microplate for 15 seconds, the plate reader began taking the kinetic readings of the fluorescence changes at 1 min intervals for 90 min. The area under the fluorescence decay curve (AUC) (excitation 485 nm, emission 528 nm) was used to calculate the antioxidant capacity;  $AUC = 0.5 + (R_2/R_1) + (R_3/R_1) + (R_4/R_1) + \dots + (R_n/R_1)$ , where  $R_1$  corresponded to the fluorescence reading at the initiation of the reaction and  $R_n$  was the fluorescence reading at the  $n$ th minute. The net AUC was determined by subtracting the AUC of the blank from that of a sample or standard. A standard curve for the Trolox was then obtained by plotting the net AUC of the series of

Trolox standards versus their concentrations ( $r = 0.99$ ). Trolox equivalent (TE) of each sample was calculated by interpolating the net AUC of sample against the Trolox standard curve. The ORAC values of the extracts, analyzed in triplicates, were averaged and expressed as  $\mu\text{mol TE/g db}$ .

### *Cell Culture*

Non-malignant colon myofibroblast CCD-18Co cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere and cultured in accordance with manufacture's recommendation. Myofibroblast are believed to be key players in inflammatory response in intestine due to their capacity to co-express and generate prostaglandin, which plays an important role in intestinal epithelial proliferation, differentiation, and inflammation through secreting growth factors and cytokines (91), therefore, the CCD-18Co cell model has clear relevance to inflammatory response in intestine.

### *Cell Proliferation Assay*

The MTT assay was performed to investigate effect of various treatments on cell proliferation. This was to establish the range of concentrations to use in subsequent assays (83).

Cells were plated (5000 cells/well) onto a 96-well plate and incubated for 24 h (at 37 °C/ humidified 5% CO<sub>2</sub> atmosphere) to allow cells to stabilize and attach onto the bottom of the wells. Cells were treated with six different concentrations (0.01-100 µg/ml) of sorghum- cowpea extract mixtures in different ratios (1:3-3:1) for 48 h. Extracts from samples were also tested separately. In an additional experiment, CCD-18Co cells were treated with four different concentrations (0.01-1 µM) of pure apigenin (dominant flavonoid in white sorghum) and quercetin (prevalent flavonoid in white cowpea) mixtures in different ratios (1:3-3:1) for 48 h. Each treatment level used 3 wells and each well received 100 µL of growth medium + treatment while control cells (0 µg/mL) received 100 µL of growth medium containing 0.1% DMSO only. All treatment levels had also 3 wells as a blank, containing 100 µL of growth media with the treatment level without cells. After incubation, viable cells were determined using the MTT assay kit that measures mitochondrial activity (Manassas, VA). The MTT reagent (15 µL) was added to each well and incubated for 4 h at 37 °C. 100 µL of solubilizing/stop solution were added to dissolve the purple crystals for 2 h. Absorbance was read at 570 nm. Blank reading (15 µL of MTT reagent plus 10 µL of solubilizing/stop solution) was subtracted from each well. Relative Growth (%) was calculated using the following formula: Relative Growth (%) = (Absorbance at 570 of test sample – blank – sample blank)/Absorbance at 570 of control cells ×100 % (83).

### *Generation of Reactive Oxygen Species Assay*

Based on data from ABTS and ORAC assay, white sorghum extracts, containing flavones as the predominant flavonoids; (mostly apigenin and luteolin) and white cowpea extract with flavonol as the major flavonoids; (mostly quercetin glycosides) were used to test interactive effects of sorghum-cowpea flavonoids combinations on intracellular ROS operation in different ratios (1:1, 1:3, 3:1). White sorghum and white cowpea extracts were also tested separately. Intracellular ROS production using 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR) as a probe were assessed for each treatment at different concentrations (0.01-100  $\mu\text{g}/\text{mL}$ ) as described by Meng et al (80) with slight alterations. In an additional experiment, CCD-18Co cells were exposed to apigenin-quercetin mixtures (0.01-1  $\mu\text{M}$ ) (dominant flavonoids in white sorghum and white cowpea) in different ratios (1:1, 1:3, 3:1) to determine the potential nature of interactive effect of sorghum-cowpea mixtures in reducing ROS generation. Cells were placed in black 96-well plate at a population density of 5000 cell/well for 24 h to allow cell attachment. Subsequently, the cells were incubated with treatments (0.01-100  $\mu\text{g}/\text{ml}$ ) and simultaneously, stimulated with 10  $\mu\text{g}/\text{mL}$  LPS (in 100  $\mu\text{g}$  media) for 24 h, followed by washing with PBS buffer to remove the spent the media. The cells were stained with 100  $\mu\text{L}$  DCFH (10  $\mu\text{M}$ ), placed in 37°C. After 30 min, the fluorescence signal was measured at 520 nm emission and 480 nm excitation with a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC). Relative fluorescence units (RFU) were analyzed using Omega Microplate Data Analyse Software and normalized to negative control (cells not treated with LPS and extracts).

### *Statistical Analysis*

Three replication of each treatment were performed. Analysis was using 2009 SAS (Version 9.3, SAS Inst. Inc., Cary, N.C., U.S.A.) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) was used to compare treatments means. Significant levels were defined using  $p < 0.05$ .

## **Results and Discussion**

### *Total Phenolic Composition of Sorghum and Cowpea Varieties*

The total phenolic content (TPC) of the extracts from different varieties of cowpeas and sorghum are presented in (Table 5). Significant differences ( $p < 0.05$ ) in TPC were found among the sorghum and cowpea phenotypes.

Among the cowpea extracts, the black, red, and light brown phenotypes had similar TPC values (not significantly different) (Table 5). This could be due to high amounts of anthocyanins, flavonols and flavan-3-ols in the black, red and light brown varieties respectively (92, 93). The high TPC values also correlated with high total flavonoid content reported in previous studies for the same varieties (92, 93). The white cowpea variety had lower TPC values than the other varieties, which could be due to much lower levels of flavonoids in the white varieties compared to the other samples (92, 93).

Among the three sorghum extracts, the high tannin phenotype showed the highest TPC values (Table 5), which is due to the high amounts of proanthocyanidins reported in

previous studies (12). The black sorghum also had a relatively high TPC value, likely due to its high 3-deoxyanthocyanin and flavone content (143, 144). The White variety had the lowest TPC values among the sorghums (Table 5), and which was expected (143, 144).

**Table 5:** Levels of total phenolic content (TPC) of cowpea and sorghum varieties.

Sorghum/cowpea Variety	Phenotype	TPC (mg GAE/g)
<b>IT97K-1042-3 Cowpea</b>	<b>Red</b>	162.4 ± 1.2b
<b>09FCV-CC-27M Cowpea</b>	<b>Light brown</b>	166.2 ± 5.5b
<b>IT 98K-1092-1 Cowpea</b>	<b>Black</b>	170.8 ± 12.52b
<b>09FCV-E CB27 Cowpea</b>	<b>White</b>	72.5 ± 3.76a
<b>ATX635×RTX436 Sorghum</b>	<b>White</b>	82.1± 9.96a
<b>TX430 (black) Sorghum</b>	<b>Black</b>	253.6 ± 14.8c
<b>High Tannin Sorghum</b>	<b>Red</b>	836.6 ± 29.6d

Data are expressed as means of triplicate experiments on dry weight basis. Values marked by the same letter are not significantly different ( $p < 0.05$ ). GAE= gallic acid equivalent.

#### *Interactive Effect of Sorghum-Cowpea Polyphenols on AOX Activity*

Oxygen radical absorbance capacity is based on hydrogen atom transfer (HAT) mechanisms involving competition between antioxidant and oxidant in trapping free radicals, while the TEAC is based on the electron transfer (ET) mechanism with no competition between the antioxidant and oxidant in redox reaction (8, 9). Due to the

different mechanisms, ORAC and TEAC assays were used to screen the enhanced action of sorghum-cowpea polyphenol combinations.

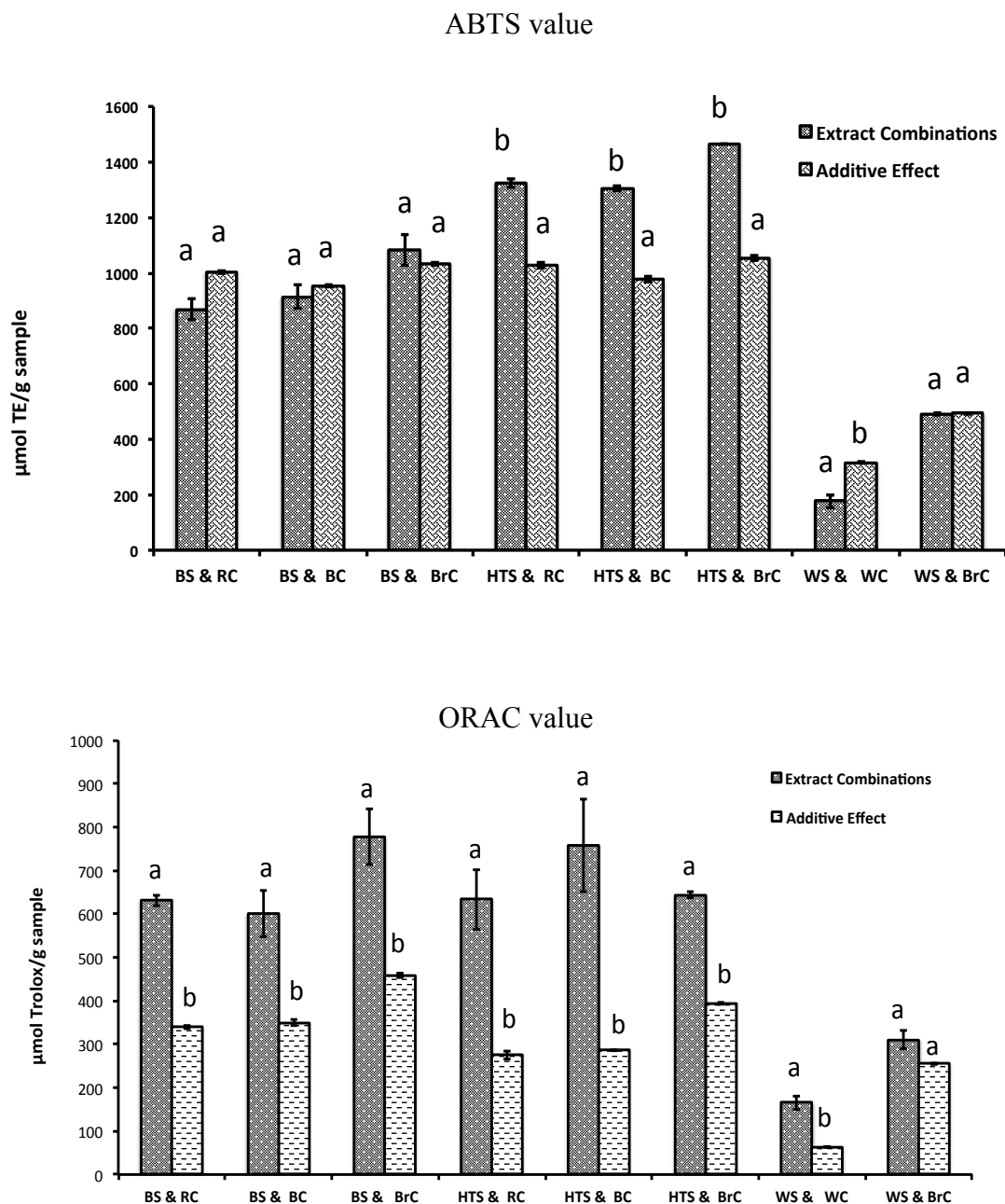
In TEAC assay, the interactive effect (enhanced action) was generally small and not consistent in all sorghum/cowpea combinations (Fig. 3). No enhanced action was observed in black sorghum combinations with black, red, and brown cowpea; whereas high tannin sorghum mixtures with the same cowpea varieties showed modest enhanced action in the range of 1.2-1.4 times higher ABTS values compare to the additive effects of individual extracts (Fig. 3). By contrast, all sorghum-cowpea mixtures showed significantly ( $p < 0.05$ ) higher ORAC antioxidant capacity than the additive affect of individual extracts (Fig. 3). Results showed a range in enhanced action values between 1.2-2.7 fold increase in sorghum-cowpea combination ORAC values relative to the additive effect. The highest enhanced action appeared in black cowpea and high tannin sorghum combination with 2.7 fold increase in ORAC antioxidant capacity versus additive effect of individual extracts ( $720 \mu\text{mol TE/g}$  versus  $270 \mu\text{mol TE/g}$ ). The second highest enhanced action was observed in white sorghum and white cowpea combination with 2.1 times higher value in ORAC compare to additive effect ( $180 \mu\text{mol TE/g}$  versus  $86 \mu\text{mol TE/g}$ ).

The end point based TEAC method showed weak and inconsistent synergy in mixtures, whereas, the kinetic based ORAC method showed strong synergy in free radical scavenging in all combinations. This is likely because ORAC measures real time protection of target mixtures against free radical by integrating AUC (area under curve)



of free radical scavenging over a 2 h period, which may be more relevant to a complex biological system.

Based on above data and what is known about the flavonoid compositions of sorghum and cowpea, we selected the white sorghum and white cowpea extracts for in depth evaluation of interactive effects of sorghum and cowpea flavonoids on ROS generation. The white sorghum-white cowpea combination had the second highest ORAC synergy (Fig. 3). They also have the simplest flavonoid profile of the sample tested (which would be discussed in the next part); thus function as a useful model to determine nature of enhanced action.



**Figure 3:** Antioxidant activities of various combinations of sorghum and cowpea varieties versus the additive effect of individual extracts measured by ABTS and ORAC methods. Error bars=  $\pm$  standard deviation ( $n = 3$ ) on dry weight basis. ( $p < 0.05$ ).

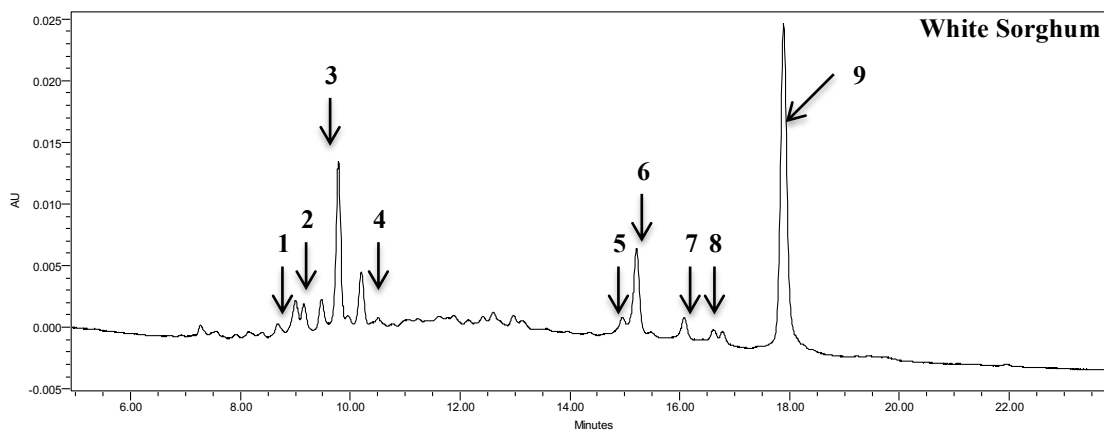
BS=Black Sorghum; HTS=High Tannin Sorghum; WS= White Sorghum.  
RC= Red Cowpea; BC= Black Cowpea; BrC= Brown Cowpea; WC= White Cowpea.

### *Characterization and Quantification of Flavonoids and Phenolic Acids in White Sorghum and White Cowpea Extracts*

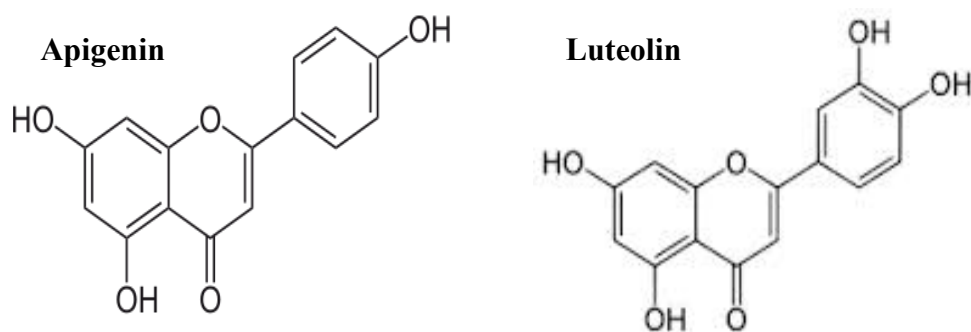
The white sorghum-white cowpea combination showed the 2.1-fold ORAC synergy, which was the second highest among the other sorghum-cowpea combinations (Fig. 4). The differences in the structural profiles of dominant flavonoids in each seemed to be the most reasonable explanation for the synergistic effect. The flavonoid profiles of white sorghum and white cowpea extracts were this characterized in detail.

#### Identification of Flavones in White Sorghum Extract

Flavones identified in white sorghum were apigenin (dominant) and luteolin (Table 6, Figure 4).



**Figure 4:** Reverse-phase UPLC chromatograms of white sorghum extract at 340 nm. Peak numbers referenced to Table 6 and 7.



**Figure 5:** Chemical structures of flavones detected in white sorghum extract.

**Table 6:** Identification of flavones in white sorghum extract (monitored at 340 nm) based on UPLC retention time ( $t_R$ ), UV-*vis* spectroscopic characteristics ( $\lambda_{max}$ ), and MS-MS/MS spectroscopic pattern.

Peak No.	$t_R$ (min)	$\lambda_{max}$ (nm)	[M-H] ( $m/z$ )	MS/MS Fragments ( $m/z$ )	Proposed Identification	White Sorghum
7	16.087	343	285	133	Luteolin	$0.448 \pm 0.091$
9	17.894	337	269	117, 119	Apigenin	$5.03 \pm 0.0298$
Total Flavone						$5.478 \pm 0.1208$

Ionization was performed in the negative mode. Peak numbers are referenced to Figure 4. Contents (mg/g extract) of flavones in white sorghum extract. All values are expressed as mean  $\pm$  SD on two separate runs. Peak values are referenced to Figure 4.

**Peak 7** ( $t_R=16.087$  min,  $\lambda_{max}=343$ ) had a [M-H]<sup>-</sup> at  $m/z$  285. The MS/MS fragmentation pattern showed one dominant ion at  $m/z$  133, which matched the Retro-Diels-Alder fragment of C-ring. Peak 7 was identified as luteolin, based on authentic standard.

**Peak 9** ( $t_R=17.894$  min,  $\lambda_{max}=337$ ) had a [M-H]<sup>-</sup> at  $m/z$  269. The MS/MS fragmentation pattern included 117 and 119, which reflected the Retro-Diels-Alder fissions of C-ring. Based on authentic standard, Peak 9 was identified as apigenin.

The presence of luteolin and apigenin has been reported in different sorghum varieties (31, 33, 144, 145).

#### Identification of Phenolic Acids and Esters in White Sorghum Extract

**Table 7:** Identification of phenolic acids and their esters in white sorghum extract (monitored at 340 nm) based on UPLC retention time ( $t_R$ ), UV–vis spectroscopic characteristics ( $\lambda_{max}$ ), and MS-MS/MS spectroscopic pattern.

Peak No.	$t_R$ (min)	$\lambda_{max}$ (nm)	[M-H] ( $m/z$ )	MS/MS Fragments ( $m/z$ )	Proposed Identification	White Sorghum
1	8.67	316	415	253, 179, 161	Caffeoylglycerol- <i>O</i> -glucoside	$0.957 \pm 0.551$
2	9.00	325	253	179, 162	2- <i>O</i> -Caffeoylglycerol	$1.578 \pm 0.083$
3	9.78	325	253	179, 162	1- <i>O</i> -Caffeoylglycerol	$6.476 \pm 2.091$
4	10.76	294	356	193	Ferulic acid- <i>O</i> -glucoside	$0.595 \pm 0.391$
5	14.95	327	415	253, 179, 161	Dicaffeoylglycerol	$1.088 \pm 0.749$
6	15.21	325	415	253, 179, 161	Dicaffeoylglycerol	$5.785 \pm 1.565$
8	16.62	315	399	253, 235, 179, 163	<i>p</i> -Coumaroyl-caffeoyl-glycerol	$0.564 \pm 0.194$
Total phenolic acids and esters						$17.043 \pm 5.624$

Ionization was performed in the negative mode. Peak numbers are referenced to Figure 4. Contents (mg/g extract) of phenolic acids and their esters in white sorghum extracts. <sup>a</sup>All values are expressed as mean  $\pm$  SD on two separate runs. <sup>b</sup>Based on molar extinction coefficient of caffeic acid. <sup>c</sup>Based on molar extinction coefficient of ferulic acid. <sup>d</sup>Based on molar extinction coefficient of coumaric acid.

**Peak 1** ( $t_R$ =8.678 min,  $\lambda_{max}$ =316) had a [M-H]<sup>−</sup> at  $m/z$  415. The MS/MS fragmentation pattern showed a dominant ion 253, which reflected the loss of hexose group (M-162 amu). The fragments of 179 and 161 matched the caffeonylglycerol

fragmentation pattern. Therefore, Peak 1 was identified as caffeoylglycerol-*O*-glucoside. This compound has been only detected in white sorghum (144, 145).

**Peak 2** ( $t_R=9.008$  min,  $\lambda_{max}=325$ ) had a  $[M-H]^-$  at  $m/z$  253. **Peak 3** ( $t_R=9.784$  min,  $\lambda_{max}=325$ ) had the same  $[M-H]^-$  at  $m/z$  253. The MS/MS fragmentation patterns of these two compounds were the same, which included a dominant ion at  $m/z$  162 and ion of  $m/z$  179. Both of the fragments matched fragmentation pattern of caffeoylglycerol (Table 7). Caffeonyl moiety substitutes at 2-*O*-position have reported to elute earlier than the substitutes at 1-*O*-position (78). Therefore peak 2 was identified as 2-*O*-Caffeoylglycerol and peak 3 as 1-*O*-Caffeoylglycerol. The presence of Caffeoylglycerol as a dominant phenolic acid has been reported in several sorghum varieties such as red sorghum (33, 126, 144).

**Peak 4** ( $t_R=10.767$  min,  $\lambda_{max}=294$ ) had a  $[M-H]^-$  at  $m/z$  355. The MS/MS fragmentation patterns of these compounds included only one dominant ion at  $m/z$  193, which corresponded to the fragmentation pattern of ferulic acid-*O*-glycoside (M-162 amu, loss of a hexose unit). Peak 4 was identified as Ferulic acid-*O*-glucoside, which was previously detected in red (TX2911), lemon yellow (SC748-H) and white sorghum (31, 144, 145).

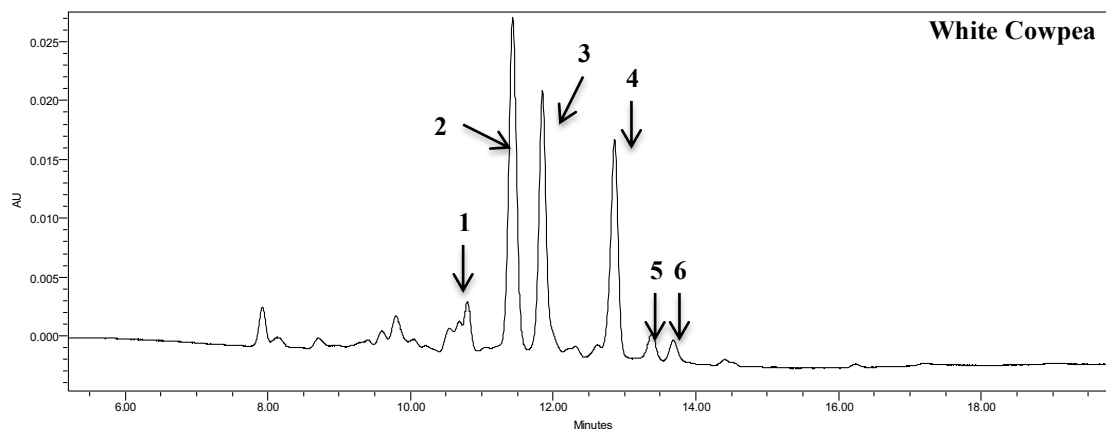
**Peak 5** ( $t_R=14.958$  min,  $\lambda_{max}=327$ ) had a  $[M-H]^-$  at  $m/z$  253. **Peak 6** ( $t_R=15.218$  min,  $\lambda_{max}=325$ ) had the same  $[M-H]^-$  at  $m/z$  415. The MS/MS fragmentation patterns of these two compounds were identical, which included a dominant ion at  $m/z$  253 (M-162 amu, loss a hexose unit) and ions of  $m/z$  179 (M-162 amu -74 amu) and  $m/z$  135 (M-162 amu -74 amu - 44 amu) which corresponded to the fragments of caffeoylglycerol,

caffeoyl and COOH. Both of the fragments matched fragmentation pattern of caffeoylglycerol (Table 7) (77). Hence, both Peaks were identified as dicaffeoylglycerol. The differences in their retention times could be due to differences in their substitution patterns such as 1,3-, 1, 2- and 2, 3-dicaffeoylglycerols. The presence of dicaffeoylglycerol has been reported in red sorghum (126, 144).

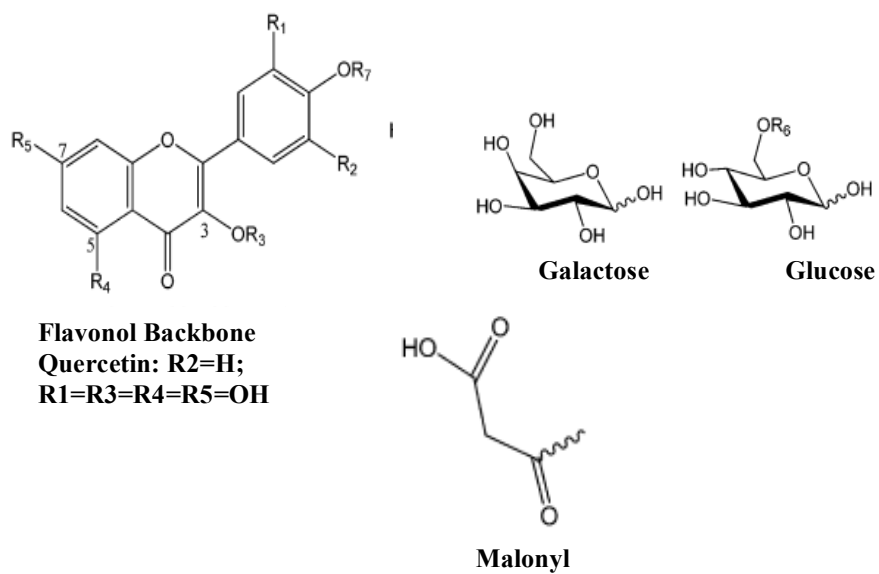
**Peak 8** ( $t_R=16.620$  min,  $\lambda_{max}=315$ ) had a  $[M-H]^-$  at  $m/z$  399. The MS/MS fragmentation patterns of these compounds included only one dominant ion at  $m/z$  253, which corresponded to the fragmentation pattern of caffeoylglycerol (M-162 amu, loss of a hexose unit) (Table 6), followed by  $m/z$  179 and  $m/z$  163 which matched the fragments of caffeoyl and coumaroyl units (144, 145).  $C_3H_5O$  is the ion that matched the backbone structure of a glycerol with no substituted COO. Therefore, Peak 8 was identified as *p*-coumaroyl-caffeoyl-glycerol, which was previously detected in red sorghum (126).

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## Identification of Flavonols and Their Derivatives in White Cowpea Extract



**Figure 6:** Reverse-phase UPLC chromatograms of white cowpea extract at 360 nm. Peak numbers referenced to Table 6.



**Figure 7:** Chemical structures of flavonol derivatives detected in white cowpea extract.



**Table 8:** Identification of flavonols and their derivatives in white cowpea extract (monitored at 360 nm) based on UPLC retention time ( $t_R$ ), UV–vis spectroscopic characteristics ( $\lambda_{max}$ ), and MS-MS/MS spectroscopic pattern.

Peak No.	$t_R$ (min)	$\lambda_{max}$ (nm)	[M-H] ( $m/z$ )	MS/MS Fragments ( $m/z$ )	Proposed Identification	White Sorghum
1	10.80 0	354	787	301, 179	Quercetin-3- <i>O</i> -triglucoside	$0.441 \pm 0.026$
2	11.43 2	354	625	301, 179, 151	Quercetin-3- <i>O</i> -galactosylglucoside	$2.755 \pm 0.857$
3	11.85 3	355	625	301	Quercetin-3- <i>O</i> -diglucoside	$2.056 \pm 0.844$
4	12.86 3	354	625	393, 301	Quercetin-3- <i>O</i> -malonylrhamnoside	$1.806 \pm 0.911$
5	13.38 8	354	463	301, 179, 151	Quercetin-3- <i>O</i> -galactoside	$0.185 \pm 0.024$
6	13.69 2	355	463	301, 179, 151	Quercetin-7- <i>O</i> -glucoside	$0.081 \pm 0.016$
Total Flavonols						$7.324 \pm 2.678$

Ionization was performed in the negative mode. Peak numbers are referenced to Figure 6. Contents (mg/g extract) of flavonols and their glycosides in white cowpea extracts. <sup>a</sup>All values are expressed as mean  $\pm$  SD on two separate runs. <sup>b</sup>Based on molar extinction coefficient of quercetin and quercetin-3-*O*-rutinoside. Peak values are referenced to Figure 6.

**Peak 1** ( $t_R$ =10.800 min,  $\lambda_{max}$ =354) had a [M-H]<sup>−</sup> at  $m/z$  787. The MS/MS fragmentation patterns included one ion at  $m/z$  301, suggesting loss of 3 glycosyl unit at the same position (M-486 amu) (Table 8), followed by  $m/z$  179 matched fragmentation pattern for quercetin (21, 25, 84, 92). Since the MS/MS fragmentation pattern showed the cleavage of glycosyl units at the glucosidic bonds between the flavylum ring and the sugars attached to it and the most common glycosylation position is usually C-3 (41, 55), this compound is identified as quercetin-3-*O*-triglucoside, which was previously reported in black and white cowpea varieties.

**Peak 2** ( $t_R$ =11.432 min,  $\lambda_{max}$ =355) had a [M-H]<sup>−</sup> at  $m/z$  625. **Peak 3** ( $t_R$ =11.853 min,  $\lambda_{max}$ =355) had the same [M-H]<sup>−</sup> at  $m/z$  625. For both peaks, the MS/MS fragmentation

pattern revealed ion of  $m/z$  301 matched loss of 2-glycosyl units ((M-324 amu). Thus, both peaks were identified as quercetin-3-*O*-dihexoside. As the eluting sequence of galactosides is reported to be slightly earlier than glucosides, (25, 84), it is reasonable to assume that one of the sugar units for peak 3 is a galactose unit; while both sugars for peak 3 are glucose. The presence of quercetin-3-*O*-galactosylglucoside has been reported in several cowpea varieties such as light brown and red (92, 93). Quercetin-3-*O*-diglucoside is one of the major flavonol glycosides identified in cowpea (21, 30).

**Peak 4** ( $t_R$ =12.863 min,  $\lambda_{max}$ =354) had a  $[M-H]^-$  at  $m/z$  625, which was similar to peak 2 and 3. However, the MS/MS fragmentation patterns included one ion at  $m/z$  393, suggesting loss of a malonyldeoxyglucoside unit (M-232 amu) (Table 8). Since its MS and MS/MS ions were similar to those previously reported in literature, peak 5 was identified as quercetin-3-*O*-manoylrhamnoside. Ojwang *et al.*, 2012, 2013 reported the presence of this compound in ten cowpea varieties.

**Peak 5** ( $t_R$ =13.388 min,  $\lambda_{max}$ =354) had a  $[M-H]^-$  at  $m/z$  463. **Peak 6** ( $t_R$ =11.853 min,  $\lambda_{max}$ =355) had the same  $[M-H]^-$  at  $m/z$  463. The MS/MS fragmentation pattern showed ion of  $m/z$  301 corresponding to loss of one glycosyl unit ((M-162 amu) from quercetin as the parent ion for both peaks. Thus, both peaks were proposed to be *O*-glycosyl derivatives of quercetin. Since the glycosylation type and position affect the retention time and UV absorption (25) and galactoside derivatives elutes earlier than glucoside derivatives, peak 5, based on shorter retention time and lower UV absorption maximum compared to peak 6, was identified as quercetin-3-*O*-galactoside, which has been previously reported in cowpea (30, 92). Peak 6 was identified as quercetin-3-*O*-

glucoside, which is one of the dominant flavonol glycosides identified in cowpea (21, 30).

According to Table 6 and Table 8, it is obvious that white sorghum and white cowpea contain distinctly different flavonoids. The flavones, apigenin and luteolin are the dominant flavonoids in white sorghum; whereas flavonols, glycosides of quercetin are dominant flavonoids in white cowpea. The differences in the structural profiles of dominant flavonoids in each may provide complementary bioactive properties in sorghum-cowpea mixtures versus the individual extracts.

#### *Effect of Relative Proportions of Sorghum-Cowpea Extracts on Magnitude of Sorghum-Cowpea Flavonoid Mixtures Synergy*

One of the factors that can impact magnitude of synergy is the relative proportion of compounds of interest. Thus, the antioxidant activities of white sorghum-white cowpea mixtures were tested in three different ratios (1:1, 1:3, 3:1) to determine the possible impact of relative proportion of extracts (10 µg extract/mL) on the magnitude of synergistic effect. The white sorghum-white cowpea combination in 1:1 ratio revealed 2.1 times higher ORAC value than the additive effect, the mixture in 1:3 ratio showed 1.8-fold increase; whereas in 3:1 ratio a showed 3.2 higher antioxidant activity versus the additive effect of individual compounds (Fig. 8. A). These results demonstrated that the ratio of antioxidant compounds, not just content, significantly affects the magnitude of synergistic antioxidant effect in sorghum-cowpea mixtures.

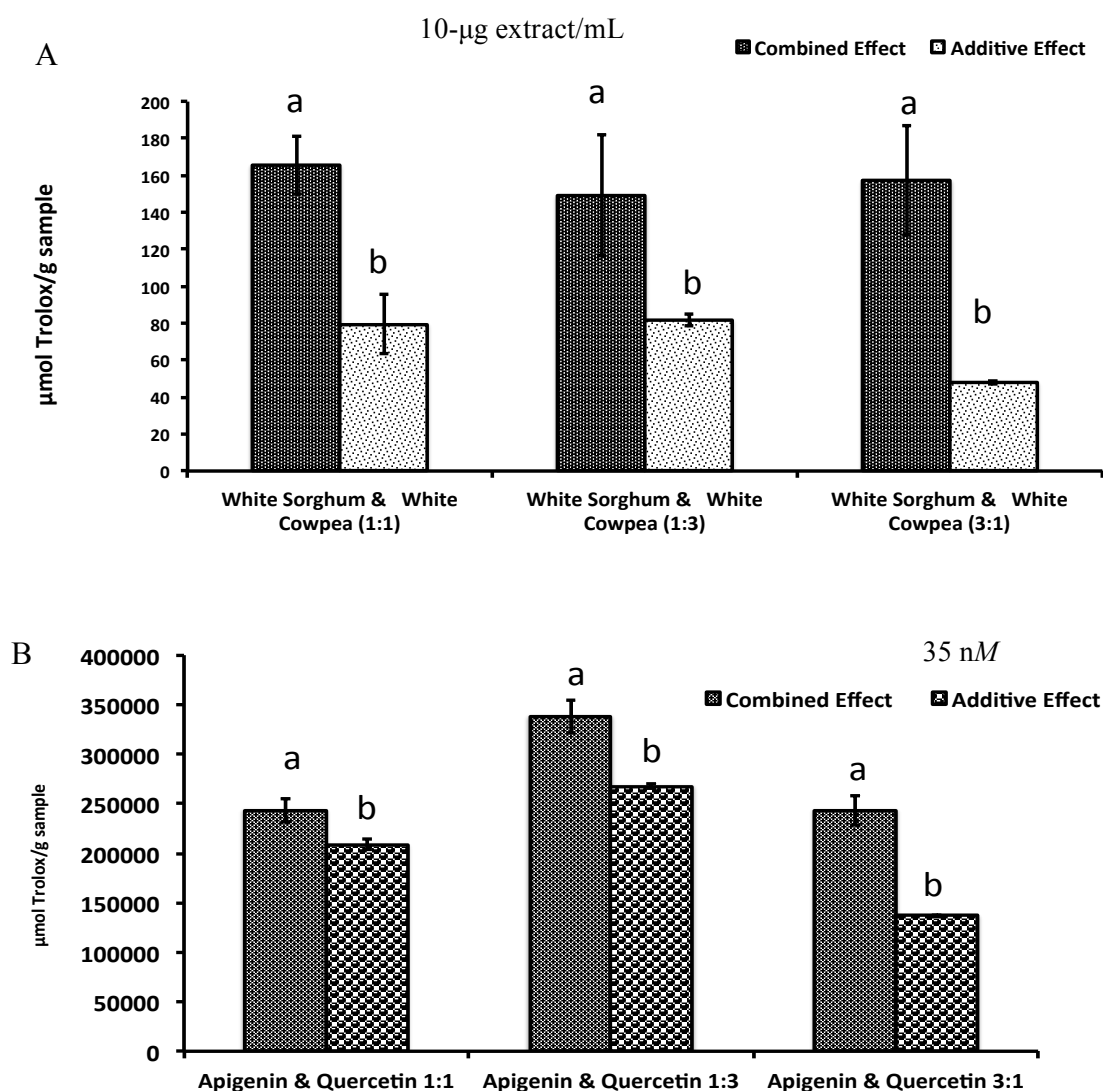
### *Interactive Effect of Apigenin-Quercetin on AOX Activity*

Apigenin was the most abundant flavonoid in white sorghum ( $5.4 \pm 0.12$  mg/g extract) and quercetin glycosides were the most dominant flavonoid in white cowpea ( $7.3 \pm 2.6$  mg/g extract). Therefore, we tested effect of combining apigenin and quercetin on AOX activity to determine the potential contribution of flavones and flavonols ratios on the synergistic antioxidant effect of white sorghum-white cowpea mixtures.

The apigenin-quercetin combination (35 nM) in 1:1 ratio revealed 1.3 times higher value in ORAC than the additive effect, followed by the mixture with 1:3 ratio (1.4-fold increase) and 3:1 ratio (1.8-fold increase) higher antioxidant activity versus the additive effect of individual compounds (Fig.8. B).

The magnitude of synergistic antioxidant effect was dependent on the relative proportions of each antioxidant compounds in both sorghum-cowpea and apigenin-quercetin mixtures and the mixture in 3:1 ratio revealed the most potent synergistic scavenging activity in both sets of treatments. These results suggest that the combinations with high ratio of apigenin showed strongest synergy in both sets of extracts. The synergistic trend was not similar in sorghum-cowpea and apigenin-quercetin mixtures. For example: the synergistic trend in sorghum-cowpea combinations was in the order of  $3:1 > 1:1 > 1:3$ ; while for the apigenin-quercetin mixtures the order was  $3:1 > 1:3 > 1:1$ . Changing the ratios of sorghum-cowpea mixtures alter concentrations of relative compounds, thus altering interactive effects of antioxidant compounds. The most important finding, however is that the mixtures were consistently more potent than the additive effect with synergistic effect of between 1.3-3.2 fold

observed, and optimum synergy was observed in the combinations with higher ratios of apigenin.



**Figure 8:** Effect of relative proportions of sorghum-cowpea extracts (10  $\mu$ g extract/mL) (**A**) and apigenin-quercetin (35 nM) (**B**) on magnitude of synergy using the ORAC method. Error bars=  $\pm$  standard deviation ( $n = 3$ ) on dry weight basis. ( $p < 0.05$ ).

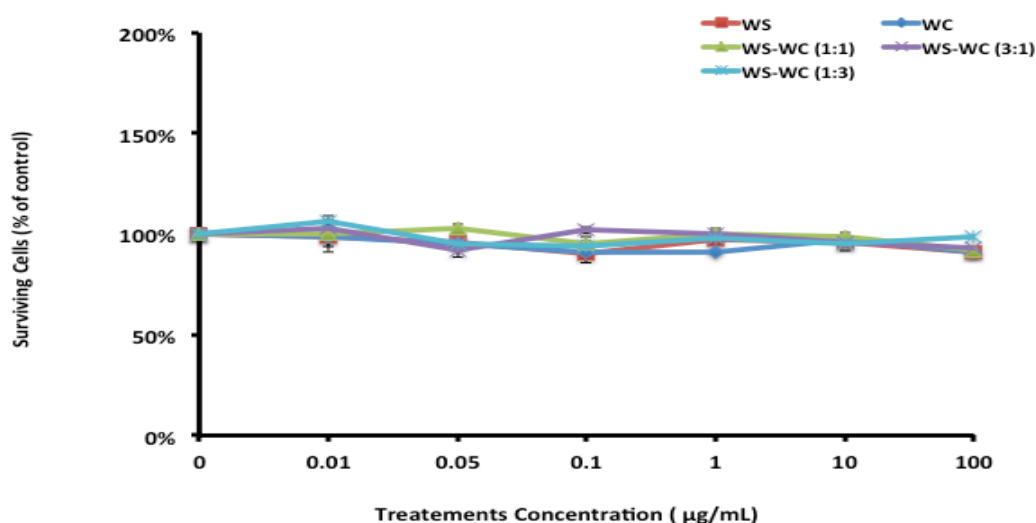
Although apigenin is the dominant flavonoid in white sorghum and quercetin glycosides are major flavonoids in white cowpea (Table 6, Table 8), the enhanced action apparent in white sorghum-white cowpea mixture was much stronger than the apigenin-quercetin mixture in all the ratios. For example: In the white sorghum-white cowpea mixture in 3:1 ratio, the enhanced action was 3.2 times higher than additive effects; whereas the apigenin-quercetin mixture in 3:1 ratio was 1.8 times higher than additive effect. A likely explanation is that in a natural crude extract like sorghum and cowpea, small quantities of other antioxidants may favorably interact to induce higher antioxidant capacity. Thus, using isolated/purified compounds in order to predict magnitude the bioactivity of flavonoids in foods may not be valid.

Apigenin is a natural subgroup of flavones. Quercetin is a natural subgroup of flavonols. Apigenin and quercetin due to the differences in functional groups do not perform similarly. Conjugation between the 3 hydroxyl group in C ring with the 4-oxo (carbonyl group) in quercetin is the key difference between sorghum and cowpea flavonoids which improves delocalization electron from C ring (69, 74). The existence of both flavones and flavonols in the same system may results in enhanced action in white sorghum-white cowpea mixture. For example: In ORAC assay, one group (in our case quercetin) may acts more rapidly and the other one (apigenin) acts slowly, which results in providing better overall protection over a two hour period than structurally similar compounds. This may explain why end point ABTS assay did not detect synergy compared to the kinetic ORAC assay.

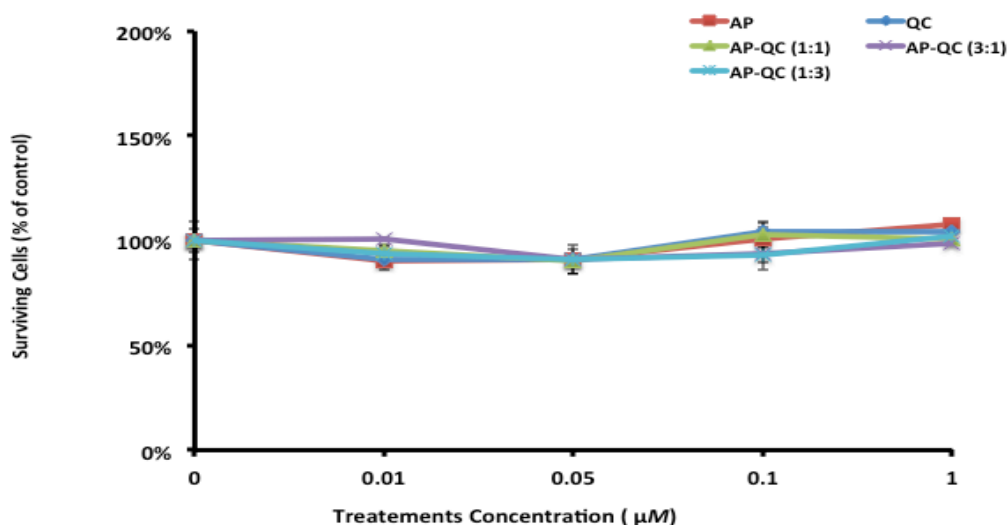
*Effect of Sorghum-Cowpea Extracts on ROS Generation in LPS Induced Non-Cancer CCD-18Co Cells*

Cell Proliferation Assay

Overall, all the tested sorghum-cowpea extracts concentrations (0.01-100  $\mu\text{g/mL}$ ) and apigenin-quercetin concentrations (0.01-1  $\mu\text{M}$ ) did not affect CCD-18Co cell growth after 48 h of incubation ( $> 85\%$  cells surviving) (Fig. 9 and 10). Therefore, extract concentrations within a dose range of (0.01-1  $\mu\text{g/mL}$ ) and (0.01-1  $\mu\text{M}$ ) were used in the subsequent assays to assess the antioxidant and anti-inflammatory properties of sorghum-cowpea and apigenin-quercetin mixtures.



**Figure 9:** Effect of sorghum and cowpea extract mixtures on proliferation of non-cancer CCD-18Co cells. Cells were treated with various concentrations of sorghum-cowpea extracts in different ratios and assessed after 48 h incubation. Values are means  $\pm$  SD ( $n = 3$ ).



**Figure 10:** Effect of apigenin and quercetin mixtures on proliferation of non-cancer CCD-18Co cells. Cells were treated with various concentrations of apigenin-quercetin mixtures in different ratios and assessed after 48 h incubation. Values are means  $\pm$  SD ( $n = 3$ ).

White sorghum-white cowpea combination extracts were tested to determine the potential interactive effects of sorghum-cowpea flavonoids in protecting the non-malignant CCD-18Co cells from LPS-induced ROS generation.

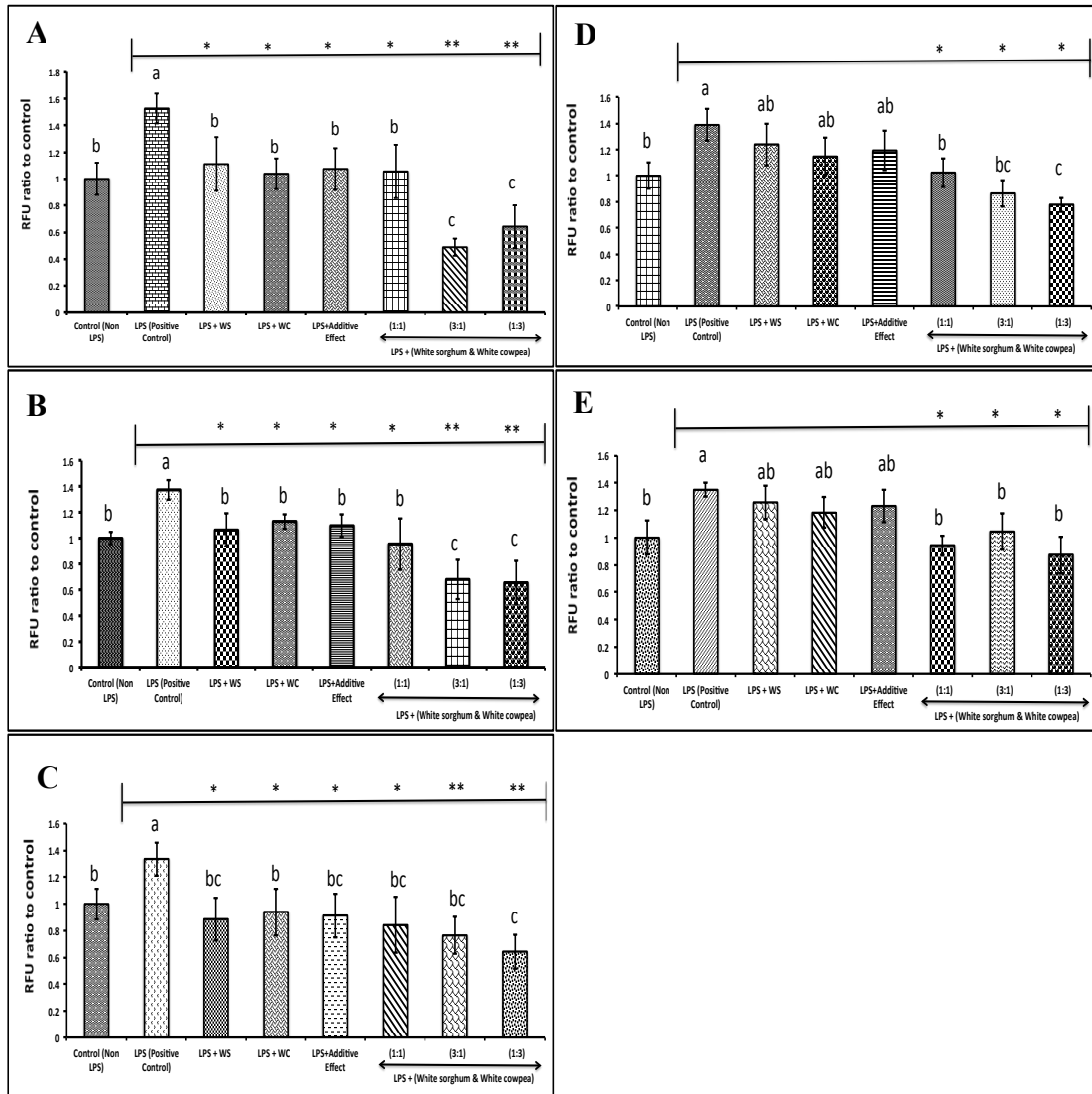
Results revealed that the protection of CCD18 cells against ROS by sorghum-cowpea mixtures was dose dependent (within 0.01 to 100  $\mu\text{g}$  extract/mL). All white sorghum-white cowpea combinations in different ratios showed strong synergistic effect in reducing ROS generation compared to the additive effect at all the tested concentrations (Fig. 11). For example: the sorghum-cowpea combination in 1:3 ratio displayed more effective reduction in ROS generation (52%) compared to the additive effect (23%) at (1.0  $\mu\text{g}/\text{mL}$ ) (Fig 11. C).



Significant difference was observed in the magnitude of synergy among the ratios at each concentration. For instance: at (10 µg/mL) concentration, sorghum-cowpea mixture in 1:3 ratio showed stronger ROS quenching (53% reduction) compared to the mixture in 1:1 ratio (31% reduction) relative to the positive control (fig. 11. B).

Ranjbar *et al.*, 2014 reported that the combinations of rosemary and oak (50 µg/mL) in 2:1, 2:3, 3:1, 3:2 and 4:1 showed significant synergistic antioxidant activity (1.5-2 times); whereas the combinations in 1:1, 1:2, 1:3, and 1:4 showed antagonistic or additive effect. These results demonstrated that change in ratios of combined rosemary-oak extracts could vary their antioxidant activity interactions from synergism to antagonistic or additive effect (107). In our study, all the mixtures showed synergistic effect, but the magnitude of synergy was different. Our results suggest that changing the ratios of the sorghum-cowpea mixtures alter concentrations of relative antioxidant compounds, thus an increase or decrease in the concentration of one or more compounds may influence the interactions and change the magnitude of synergy.

The minimum effective concentration that showed significant ROS mitigation for additive effect of individual extracts was in 1.0 µg/mL (Fig. 11. C); whereas the sorghum-cowpea mixtures significantly ( $P < 0.05$ ) reduced ROS generation at 0.05 µg/mL (Fig. 11. E). This represents a twenty times higher minimum effective dose for additive effect relative to the synergistic effect, suggesting a twenty fold better ROS quenching power in combinations.



**Figure 11:** Effect of sorghum-cowpea extract mixtures on LPS-induced ROS generation in non-cancer CCD-18Co cells. Cells were pretreated with sorghum-cowpea mixtures extracts **A**: (100  $\mu\text{g/g}$ ), **B**: (10  $\mu\text{g/g}$ ), **C**: (1  $\mu\text{g/g}$ ), **D**: (0.1  $\mu\text{g/g}$ ) and **E**: (0.05  $\mu\text{g/g}$ ) in different ratios (1:3-3:1) for 24 h. Values are  $\pm$  SD,  $n = 3$ ; (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to non-LPS treated control.

Many studies have investigated the synergistic antioxidant effects between phytochemicals (15, 48, 60, 78, 98, 135). A few of the studies have reported synergistic effects in protection of endothelial cells against ROS generation by phytochemical mixtures, but these studies are still limited to purified polyphenol mixtures at higher concentrations compared to our study (97, 109, 116, 121). The *Scutellaria baicalensis* (Chinese herbal medicine) (100 µg/mL) and grape seed proanthocyanidins (GSPE) (10 µg/mL) mixture in equal ratios synergistically scavenge ROS production (38% reduction) versus the additive effect of individuals (13% reduction) (121); while the mechanisms and the potential compounds that contribute to the apparent synergistic effect was not elucidated. Ravit *et al.*, 2009 reported that the combination of a natural antioxidant extract from spinach leaves (2.4 mg/mL) with 50µM of ferulic acid; caffeic acid and EGCG were more effective (2.2, 3, and 2.6 fold respectively) in scavenging ROS production versus the additive effect of individual components (109). Another study reported combination index value for binary combinations of (quercetin: pterostilbene), (kaempferol: pterostilbene) and (quercetin: kaempferol) (1.56 µM) in 1:1 ratio was significantly less than 1.0 ( $0.57 \pm 0.09$ ), ( $0.56 \pm 0.11$ ) and ( $0.43 \pm 0.07$ ) respectively. This indicates that combinations of compounds at lower concentrations versus the individual compounds exhibit synergistic activity in protecting HepG2-C8 cell line against oxidative stress (116).

Our study is the first to report synergistic protection of endothelial cells against ROS generation by natural complementarily flavonoid mixtures as found in foods with practical relevance to diet. The co-administration of distinctly different flavonoid classes

present in white sorghum-white cowpea mixtures resulted in synergy in their capacity to mitigate generation of ROS in non-cancer cells. Results from this study suggest that sorghum-cowpea combinations may protect cellular components (e.g. DNA, etc) from oxidative damage at lower levels of intake than can be predicted by testing individual commodities.

Apigenin as the most abundant antioxidant flavonoid in white sorghum ( $5.5 \pm 0.12$  mg flavones/g extract) is known to exert antioxidant property in free radical scavenging systems and protect cells from oxidative damage by decreasing ROS levels. For example: Apigenin decreased ROS [hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and superoxide anion ( $\text{O}_2^-$ )] levels in a dose dependent manner ( $9\text{-}74\text{ }\mu\text{M}$ ) by almost 60% and 40% respectively in *H. pylori* ATCC 700824-infected MKN45 cells (136). In another study, apigenin inhibited formation of hydroxyl radical and superoxide anion in a concentration-dependent manner ( $37\text{-}186\text{ }\mu\text{M}$ ) in human peripheral blood lymphocytes (HPBL). The IC<sub>50</sub> values of apigenin against hydroxyl radical, and superoxide anion were  $111.6 \pm 4.1$  and  $101.9 \pm 5.8\text{ }\mu\text{M}$ , respectively, and the free radical scavenging capacity was comparable to ascorbic acid (16).

Quercetin derivatives, the most dominant antioxidant flavonoid in white cowpea ( $7.3 \pm 2.7$  mg flavonols/g extract) were also reported to inhibit superoxide and nitric oxide (NO) production as well as superoxide and peroxynitrite ( $\text{ONOO}^-$ ) scavenging at  $30\text{ }\mu\text{M}$  concentration in in Raw 256.7 macrophages (67). An animal study showed that administration of 25 mg quercetin /kg body weight from 1 to 18 weeks inhibited the development of DMBA-induced hamster buccal pouch (HBP) carcinomas by impairing

CYP-mediated ROS production via down-regulating the mRNA and protein expression of cytochrome P450 CYP isoforms (CYP1A1, CYP1B1) by almost 50% (106).

Thus, the combinations of apigenin, quercetin and their derivatives from sorghum and cowpea extracts probably have different mechanism of action, which resulted in synergistic mitigation of oxidative damage in the cells. To test this, apigenin and quercetin mixtures were used in this study to determine the potential contribution of the flavones and flavonols in the synergistic effect of sorghum-cowpea mixtures against oxidative damage.

#### *Effect of Apigenin and Quercetin Against LPS-Induced ROS Generation in Non-Cancer CCD-18Co Cells*

The apigenin-quercetin mixtures were more effective in protecting CCD18 cells against ROS generation than the additive effect of individual extracts (Fig. 12), which is consistent with results on the synergistic antioxidant activity of sorghum-cowpea mixtures (Fig. 11).

Corresponding to the sorghum-cowpea mixtures results, apigenin-quercetin combinations in different ratios (1:1, 1:3, 3:1) (at similar concentrations), exhibited significantly ( $P < 0.05$ ) different inhibitory effect against ROS formation (Fig. 12). For instance: The combination in 1:3 ratio (60%) was more potent in ROS quenching compared to the combination in 1:1 ratio (42%) at 1.0  $\mu\text{M}$  (Fig.12. A).

The minimum effective dose that revealed significant reduction of ROS generation for additive effect of individual extracts was (0.1  $\mu\text{M}$ ) (Fig. 12.B); whereas the apigenin-

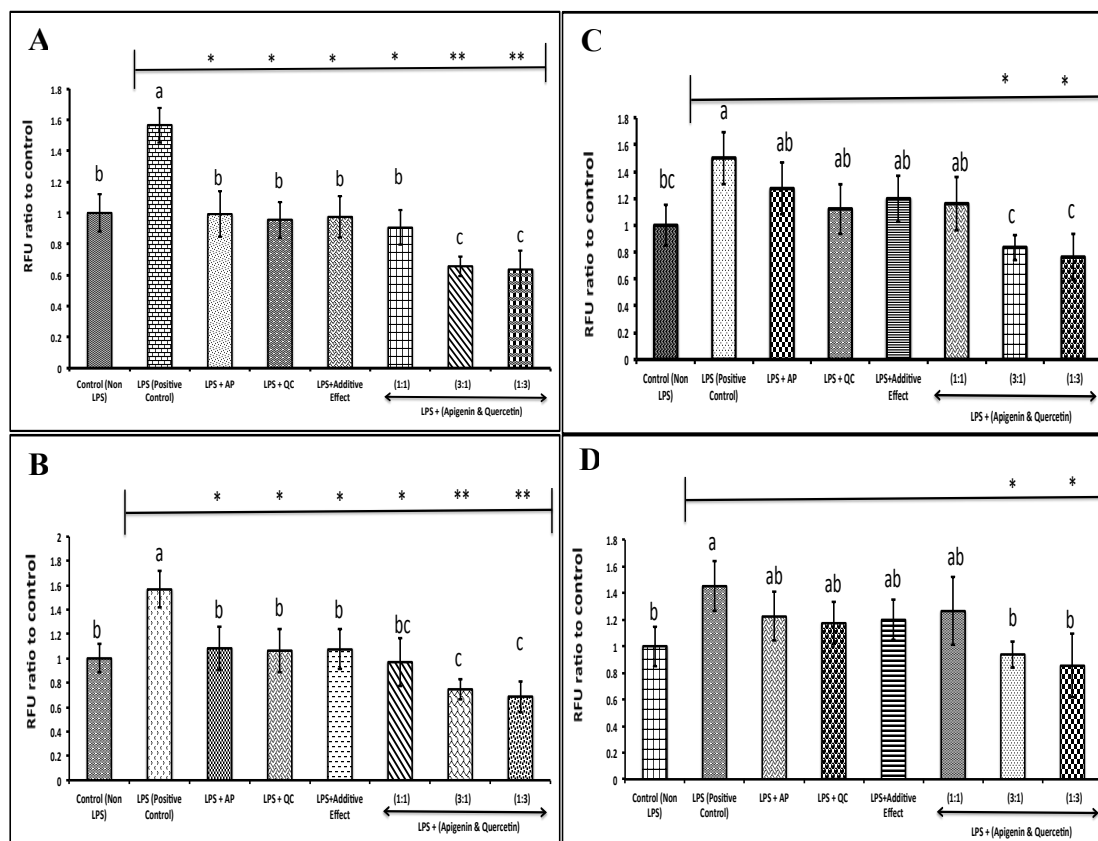
quercetin mixtures significantly ( $P<0.05$ ) protected CCD-18 cells against ROS at 0.01  $\mu\text{M}$  (Fig. 12.D) representing a 10-fold synergistic effect. These results support the hypothesis that flavone/flavonol mixtures can synergistically attenuate ROS formation.

In our study, significant differences were observed in the magnitude of synergy among the ratios in both sorghum-cowpea and apigenin-quercetin mixtures and the mixture in 1:3 ratio showed the strongest synergistic effect in both set of mixtures; whereas small non significant difference was observed between individual apigenin and quercetin in ROS scavenging. These results suggest that both apigenin and quercetin separately reduced ROS generation via different mechanisms.

The co-administration of structural complementary flavonoids (apigenin-quercetin) with different mechanism of action thus has the potential to enhance the antioxidant capacity and provide better protective effect. This supports the hypothesis that flavone/flavonol combinations are largely responsible for synergistic protective capacity against oxidative damage by sorghum-cowpea mixtures.

Consistent synergy in protecting non-cancer cells against ROS generation was observed in both sorghum-cowpea and apigenin-quercetin results, indicating the likely prevalent contribution of flavones and flavonols in synergistic effect. Although synergistic effect was observed in almost similar concentration range in sorghum-cowpea extract mixtures (0.6-120 ng flavonoid/mL) and apigenin-quercetin mixtures (2.85-285 ng flavonoids/mL), the minimum effective concentration that showed synergistic effect in sorghum-cowpea mixtures was 4.5 times lower than of apigenin-quercetin mixtures (0.6 ng/mL versus 2.85 ng/mL). These results suggest that flavone/flavonol are likely the dominant contributors to synergistic

antioxidant activity, but due to the complexity of sorghum-cowpea extracts and their multifaceted mutual interactions, it is not possible to predict the magnitude of bioactivity of natural extract mixtures by using isolated/purified compounds.



**Figure 12:** Effect of apigenin-quercetin mixtures on LPS-induced ROS generation in non-cancer CCD-18Co cells. Cells were pretreated with apigenin-quercetin mixtures extracts **A**: (1.0  $\mu$ M), **B**: (0.1  $\mu$ M), **C**: (0.05  $\mu$ M), and **D**: (0.01  $\mu$ M) in different ratios (1:3-3:1) for 24 h. Values are  $\pm$  SD,  $n = 3$ ; (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to non-LPS treated control.

## **Conclusions**

Taken together, enhanced action of white sorghum and white cowpea flavonoid combinations versus the additive effect of individual extracts was strongly exhibited in ORAC assay. The White sorghum-white cowpea combinations were able to synergistically attenuate the LPS-induced ROS generation in non-cancer CCD-18Co cells at very low concentrations that are likely achievable through diet. The combination of apigenin and quercetin significantly enhanced their action in protecting the CCD-18Co cells against ROS generation. The enhanced action in apigenin-quercetin mixtures was adequate to explain the contribution of flavones and flavonols in the synergistic action of sorghum-cowpea mixtures against oxidative stress. Differences in major types of flavonoids present in sorghum and cowpea resulted in enhanced action in their capacity to mitigate generation of ROS, which paralleled their antioxidant capacity. The magnitude of synergy was dependent on the relative proportions in both ORAC and ROS assays, indicating strategic cereal and legume combinations might be formulated for optimized benefits in chronic disease prevention.



**CHAPTER IV**

**INTERACTIVE EFFECTS OF SORGHUM AND COWPEA FLAVONOID  
MIXTURES AGAINST INFLAMMATION IN NON-MALIGNANT COLON  
MYOFIBROBLAST CCD-18CO CELLS**

**Introduction**

Chronic inflammation plays an important role in a wide variety of chronic diseases including cardiovascular disease (CVD) and cancer (58, 67). CVD and cancer are the leading causes of death in developed countries; and are growing problems in developing countries (71, 124). Epidemiological studies have established an association between whole grain and grain legume intake and reduction the risk of CVD, cancer and diabetes, which may be related to their polyphenol compositions and dietary fiber, among others (71, 76, 126).

Cereals and legumes are traditionally consumed together to overcome protein deficiency, improve food security and provide required vitamin and minerals (44, 89, 137). Among the cereal grains, sorghum is one of the major crops and plays an important role in food security of semi and small-scale farmers in Africa (8). Sorghum has been recently shown to contain high levels of polyphenols compared to other crops (8, 28). Cowpea is a stress resilient legume that provides high quality proteins to many low-income populations (100). Sorghum and cowpea are both well adapted to semi arid regions and perform better than other cereals and legumes under environmental stresses

(e.g. high heat and low moisture climate), thus they are produced and consumed together (e.g. in west Africa) (8, 85).

Recent evidence demonstrates that sorghum and cowpea also contain distinctly different flavonoids with structural profiles that may provide complementary bioactive properties (8, 92, 93). For example, the majority of monomeric sorghum flavonoids are un-substituted at position 3 of the heterocyclic ring (flavones, flavanones and 3-deoxyanthocyanidins) and exists mainly as aglycones. In cowpea, the dominant monomeric flavonoids are substituted at position 3 (glycosides of quercetin, anthocyanins and flavan-3-ols) and are mostly glycosylated (92). Additionally sorghum contains mainly large molecular weight flavan-3-ols (condensed tannins), whereas in cowpea, these compounds mostly exist as glycosylated monomers (8, 92, 93).

Flavonoids, depending on their chemical structure suppress several inflammatory mediators via diverse cellular mechanisms (58, 70, 103, 129). The majority of flavonoid classes suppress the activity of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen activated protein kinase (MAPK), which are key regulators of inflammation, resulting in expression of several pro-inflammatory cytokines and enzymes involved in chronic inflammation disorders (67). Researchers have recognized that the action of individual bioactive compounds alone do not explain the health benefits of food mixtures. Bioactive constituents are always ingested in the form of natural combinations, hence, interactions among phytochemicals such as flavonoids contribute to the ability of natural foods to protect human health and mitigate disease damage. However, the majorities of investigations on phytochemical interactions are still limited to *in-vitro* antioxidant tests

or purified polyphenol mixtures (2, 52, 54, 63, 107, 109, 121, 135). Few studies have taken advantage of structural complementarity of naturally occurring flavonoids to evaluate potential health benefits of foods. Sorghum and cowpea provide an intriguing opportunity to demonstrate the possible extent of enhanced benefits of cereal-legume mixtures. It is expected that the structurally different flavonoid classes in sorghum and cowpea will target different signaling mechanisms and induce a greater anti-inflammatory effect than flavonoids mixtures in an individual commodity. In this study, we demonstrate synergistic interaction of sorghum-cowpea flavonoids against inflammation using non-malignant myofibroblast CCD-18Co cells.

## **Materials and Methods**

### *Chemicals and Reagents*

Pure standards used in this investigation were as follows: Apigenin was from Indofine (Indofine Chemical Company, Inc., Hillsborough, NJ); quercetin was from Sigma-Aldrich Chemicals, St. Louis, MO. Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The forward and reverse pair primers (IL-6, NF- $\kappa$ B, TNF- $\alpha$  and IL-8) were obtained from Integrated DNA Technologies, Inc. (San Diego, CA). Bradford reagent was obtained from BioRad (Hercules, CA), antibodies against NF- $\kappa$ B p65, phospho-NF- $\kappa$ B p65 and  $\beta$ -actin were obtained from Cell Signaling Technology (Beverly, MA).

### *Treatments Preparation*

Whole kernel white sorghum grain and dry seed white cowpea were selected as treatments of study based on the results from Chapter 3. They showed a strong synergistic antioxidant effect in ORAC and cell model assays and have relatively simple flavonoids profiles (Fig. 1, 3, Tables. 4, 6), which is ideal for investigating the possible mechanisms of interactions. Ground white sorghum and white cowpea were individually extracted with 70% aqueous acetone 3 times (1:4 ratio, 100 g portion, 2 h then 1 hr and 1 hr duration of extraction). The supernatant from all extractions was collected by filtering through a No.1 filter paper (VWR, Radnor, PA) and combined. Organic solvent was removed by rotary evaporation (Rotovap, Büchi, Flawil, Switzerland) under vacuum at 40°C and aqueous fraction was freeze-dried to obtain powder extracts and stored at -80°C until use. The chemical composition of extracts was established and described in Chapter 3 (Tables. 4, 6).

### *Cell Culture*

Non-malignant colon myofibroblast CCD-18Co cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere and cultured in accordance with manufacture's recommendation. Myofibroblast are believed to be key players in inflammatory response in intestine due to their capacity to co-express and generate prostaglandin, which plays an important role in intestinal epithelial proliferation, differentiation, and inflammation through secreting growth factors and cytokines (102).

Therefore, the CCD-18Co cell model has clear relevance to inflammatory response in intestine. This cell model has been used effectively in the past to provide evidence of anti-inflammatory potency of polyphenols in modulating the level of inflammatory markers relevant to colonic inflammation (5, 40, 88, 91).

#### *LPS-Induced Inflammatory Assay*

Cells were seeded in 12 well plate at a population density of 100,000 cells/well, incubated at 37°C/ 5% CO<sub>2</sub> for 24 h to settle the cells. Treatments pre-dissolved in DMSO were diluted and normalized in culture medium to reach the appropriate concentrations (0.01-1 µg/mL) (< 0.2% DMSO). Since white sorghum/white cowpea mixtures showed significant reduction in ROS generation at low concentrations (0.05, 0.1, 1 µg/mL), for LPS-induced inflammatory assay, we focused on these low concentrations. The cells were subsequently treated with extracts (0.01-1 µg/mL) and simultaneously stimulated with 10 µg/mL LPS for 9 h. Messenger RNA (mRNA) were then extracted from the lysated cells and analyzed. In an additional experiment, CCD-18Co cells were exposed to apigenin-quercetin mixtures (0.05-1 µM) (dominant flavonoids in white sorghum and white cowpea) to determine the potential nature of interactive effect of sorghum-cowpea mixtures in modulating pro-inflammatory cytokines and NF-κB mRNA expression.

### *RNA Extraction and Real-Time PCR Analysis of mRNAs*

Total RNA was extracted using the Qiagen extraction kit (Qiagen Inc. Valencia, CA) based on the manufacturer's protocol. The NanoDrop<sup>®</sup> ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) were used to evaluate the quality and quantity of isolated RNA.

### *mRNA Analysis*

A Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) was used to synthesize complementary DNA (cDNA) from the isolated RNA based on the manufacturer's protocol. Real Time PCR (qRT-PCR) was performed with the SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA).

### *Primer Sequences Used for mRNA Analysis*

The sequences of primers used were as follows:

IL-8:	F:	5'-CACCGGAAGGAACCATCTCA-3'
IL-8:	R:	5'-AGAGCCACGGCCAGCTT-3'
TNF- $\alpha$ :	F:	5'-TGTGTGGCTGCAGGAAGAAC-3'
TNF- $\alpha$ :	R:	5'-GCAATTGAAGCACTGGAAAAGG-3'
NF- $\kappa$ B:	F:	5'-TGGGAATGGTGAGGTCCTCT-3'
NF- $\kappa$ B:	R:	5'-TCCTGAACTCCAGCACTCTCTTC-3'

IL-6                      F:        5' - AGGGCTCTTCGGCAAATGTA-3'

IL-6                      R:        5'-GAAGGAATGCCCATTAAACAACAA-3

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) as a transcription factor is the key regulator of inflammation, leading to expression of pro-inflammatory cytokines, which are associated in carcinogenesis development and other chronic inflammation disorders (90, 108, 129). Pro-inflammatory cytokines such as interleukins (IL-6) and tumor necrosis factor (TNF- $\alpha$ ) are substances produced by immune cells (e.g. mast cells, macrophages and neutrophils). TNF- $\alpha$  is considered as a master switch from inflammation to cancer, which is also known to activate NF- $\kappa$ B in immune system (67, 103). Thus, inhibition either one by flavonoids may help to prevent transcription of other pro-inflammatory molecules. The activation of the NF- $\kappa$ B pathways leads in phosphorylation to phosphorylated-NF- $\kappa$ B ( $\rho$ -NF- $\kappa$ B), releasing NF- $\kappa$ B and allowing it to activate expression of inflammatory mediators (90). Therefore, higher  $\rho$ -NF- $\kappa$ B/NF- $\kappa$ B ratios represent higher inflammatory response. IL-8 as a pro-inflammatory cytokine/chemokine was included in this study due to the function of promoting phagocytosis of neutrophils, which results in ROS secretion (70). TNF- $\alpha$ , IL-6 and IL-8 are inducible by lipopolysaccharide (LPS) as well as NF- $\kappa$ B.

### *Protein Analysis*

Cells were seeded ( $3 \times 10^5$  cells/well) in 6-well plates and incubated for 24 h. to allow cell attachment before subjecting them to treatments at (0.1  $\mu\text{g/mL}$ ) for 24 h. Cells were harvested and cell lysates were obtained using the Pierce Ripa buffer (25mM Tris®HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) supplemented with Halt protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL.). Protein content was assessed using the Bradford reagent (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Proteins were separated on a 10 and 20% SDS-PAGE at 120 V for 1 h. and transferred to PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (27). Membranes were blocked with 5% non- fat milk in 0.1% Tween-PBS (T-PBS) solution for 1 h. and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in T-PBS overnight at 4C with gentle shaking. After 3 washing steps with T-PBS for 5 minutes each, membranes were incubated with secondary antibodies (1:2000) in 5% non-fat milk dissolved in T-PBS for 1 h. Membranes were washed three times for 15 minutes and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY). The Luminex® assay was assessed using an 11-plex AKT/mTOR phosphoprotein magnetic bead kit (Millipore, Billerica, MA) following the manufacturer's protocol. Data were analyzed using Luminex xPonent 3.0 software.



### *Statistical Analysis*

Three replication of each treatment were performed. Analysis was using 2009 SAS (Version 9.3, SAS Inst. Inc., Cary, N.C., U.S.A.) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) was used to compare treatments means. Significant levels were defined using  $p < 0.05$ .

## **Results and Discussion**

### *Effects of White Sorghum-White Cowpea Mixtures on NF- $\kappa$ B Gene and Protein Expression*

At the highest concentration tested (1.0  $\mu\text{g/mL}$ ), sorghum-cowpea extract combinations showed significantly ( $P < 0.05$ ) higher down regulation in NF- $\kappa$ B expression than the additive effect of individual extracts at mRNA level (Fig.13). Higher average reduction (85%) in NF- $\kappa$ B gene expression was exhibited by the sorghum-cowpea extract combination compared to the additive effects of individual extracts (36%) relative to the positive control (LPS-treated cells) (Fig.13). The minimum effective dose that showed significant mitigation of LPS induced NF- $\kappa$ B gene expression for additive effect of individual extracts at similar levels was (1.0  $\mu\text{g/mL}$ ); whereas the sorghum-cowpea mixtures significantly ( $P < 0.05$ ) inhibited NF- $\kappa$ B gene expression at 0.1  $\mu\text{g/mL}$  (Fig.13 and 14).

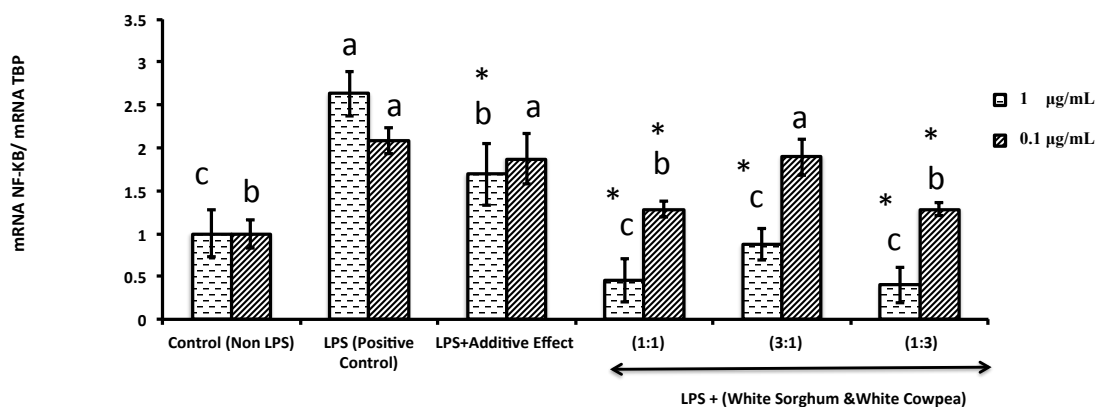
To test whether the synergistic anti-inflammatory effect were dependent on the

relative proportion of sorghum and cowpea extracts, the extracts were mixed at ratios of 1:1, 3:1, and 1:3. Sorghum-cowpea mixtures in different ratios (at similar concentrations), revealed significantly ( $P < 0.05$ ) different down regulation in NF- $\kappa$ B mRNA expression (Fig.13). For example: at 0.1  $\mu\text{g/mL}$  concentration, mixture with (1:3) ratio showed stronger inhibitory effect (40%) against NF- $\kappa$ B gene expression than the mixture with (3:1) ratio (10%). Similar trend was observed at 1.0  $\mu\text{g/mL}$  (Fig. 14).

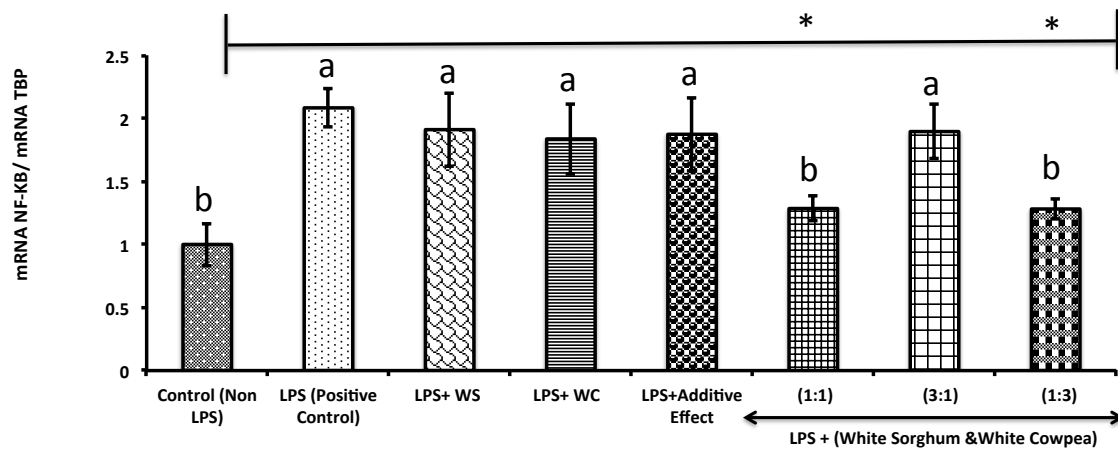
Such strong dependency of synergistic effect on relative proportions of the compounds present in each extract suggests the compounds in sorghum and cowpea act via different mechanisms. Rawat *et al.*, 2015 (43) found the best antioxidant synergy in pyrogallol: tert-butylhydroquinone (PY:TBHQ) and pyrogallol: propyl gallate (PY:PrG) combinations at 1:3 weight ratios versus the other weight ratios tested (1:9-9:1) (110). In contrast, Park *et al.*, 2011 did not observe significant differences between pure phytochemicals (luteolin and chicoric acid) mixtures at different ratios (1:1, 1:2, 1:4) in reducing NO production. They concluded that luteolin plays a central role in reducing NO, and that chicoric acid strengthens luteolin's effect in a dose dependent manner (97). No studies have reported the dependency of magnitude synergistic effect on relative proportions in natural complementarily mixtures.

In order to determine whether the inhibition of the mRNA expression of NF- $\kappa$ B by sorghum-cowpea mixtures is correlated to the reduction of protein levels, we measured NF- $\kappa$ B protein expression by western blot analysis at 0.1  $\mu\text{g/mL}$  which was the minimum effective dose that showed synergistic effect in NF- $\kappa$ B gene expression results (Fig. 15). The western blot analysis showed stronger reduction in p-NF- $\kappa$ B/NF- $\kappa$ B ratio

(60%) than the additive effect (40%) in the same treatment (Fig. 15), which indicates stronger operation of combined treatments against inflammatory response versus the individual treatments. Sorghum-cowpea mixtures in different ratios revealed significantly ( $P < 0.05$ ) different down regulation in NF- $\kappa$ B in protein level as well (Fig. 15). For example: the combination in 3:1 ratio (67%) was more effective in reducing p-NF- $\kappa$ B/NF- $\kappa$ B ratio compared to the combination in 1:1 ratio (49%) (Fig.15).

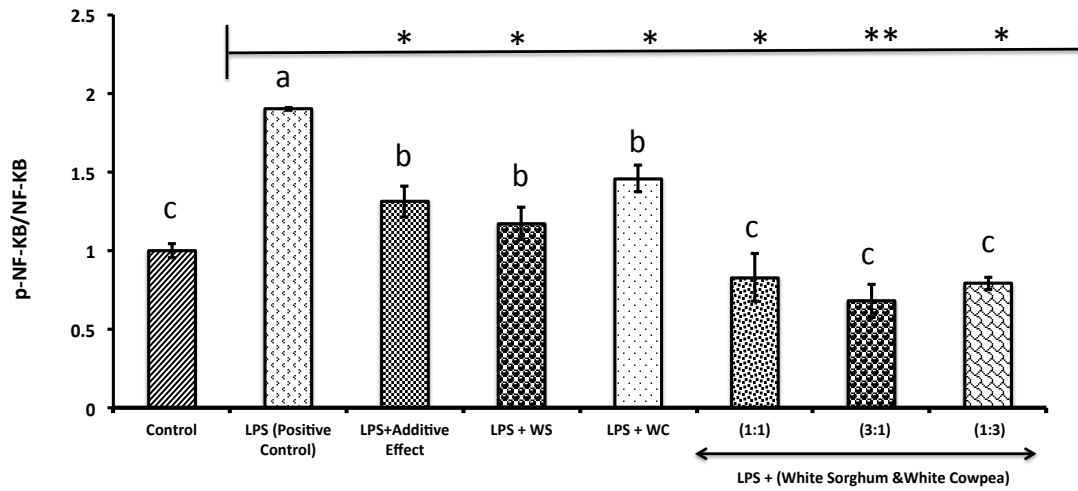


**Figure 13:** Effect of extracts of sorghum and cowpea mixtures on nuclear factor-  $\kappa$ B (NF- $\kappa$ B) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.1-1.0  $\mu$ g/mL, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

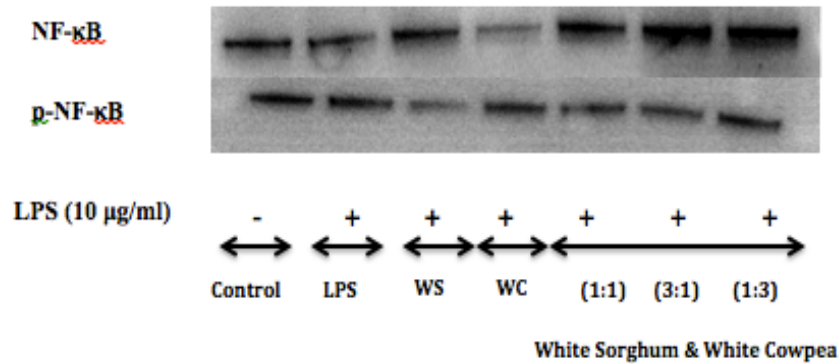


**Figure 14:** Effect of extracts of sorghum and cowpea mixtures on nuclear factor-  $\kappa$ B (NF- $\kappa$ B) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.1  $\mu$ g/mL, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

**A**



**B**



**Figure 15:** Effect of extracts of sorghum and cowpea mixtures on nuclear factor-  $\kappa$ B (NF- $\kappa$ B) protein expression in CCD-18Co cells. Ratio of CCD-18Co phospho-Nuclear Factor- $\kappa$ B / Nuclear Factor- $\kappa$ B ( $\rho$ -NF- $\kappa$ B/NF- $\kappa$ B) protein expression. Cells were treated with different (0.1  $\mu$ g/mL) sorghum and cowpea combinations for 24 h, then challenged with LPS for 24 h. Protein expression was analyzed by Western Blot, normalized with untreated control cells (**A**). Western blot analysis of  $\rho$ -NF- $\kappa$ B/NF- $\kappa$ B (**B**). Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

NF- $\kappa$ B as a critical transcription factor in inflammation pathway, was synergistically attenuated at both mRNA and protein levels by sorghum-cowpea mixtures. NF- $\kappa$ B is usually found in its inactive form bound to the inhibitor (I $\kappa$ B- $\alpha$ ) in cytoplasm, which prevents its translocation into the nucleus (91). Myofibroblasts CCD-18Co cells activated by inflammatory stimuli (proinflammatory cytokines such as IL-8 and TNF- $\alpha$ ) accelerate inhibitor (I $\kappa$ B- $\alpha$ ) phosphorylation, which leads to NF- $\kappa$ B release and its translocation into the nucleus (91). The enhanced action of white sorghum-white cowpea mixtures to down-regulate LPS-induced expression of NF-  $\kappa$ B suggest their significant role in modulating downstream signaling of NF-  $\kappa$ B pathways, which is consistent with previous studies on the anti-inflammatory effect of white cowpea flavonoids at higher (10 times) concentrations (91).

A few studies have reported synergistic anti-inflammatory effects of phytochemical mixtures in down regulating NF-  $\kappa$ B pathway (22, 48, 69, 97, 116, 140), and are mostly limited to purified polyphenol mixtures at higher concentrations compared to our study. Such high polyphenol concentrations do not mimic the low dietary consumption levels. For example: Park *et al.*, 2011 reported the co-treatment of luteolin and chironic acid was more effective in reducing I $\kappa$  $\beta$  and p65 protein expression by almost (70%) and (60%) than the individual treatments at 24  $\mu$ M concentration in LPS induced RAW 264.7 cells which resulted in synergistically down regulation of NF-  $\kappa$ B activation. The effect of ratios was not considered in this study and all the treatments were in the same (1:2 (luteolin: chironic acid)) ratio (97). An animal study by Wu *et al.*, 2015 has demonstrated that the individually mixtures of  $\beta$ -carotene (10 mg/kg) with low doses (50

mg/kg) and high doses (100 mg/kg) of quercetin synergistically increased the ratio of cytoplasm/ nucleus NF-  $\kappa$ B protein levels by almost 50% compared to the individual treatments, which indicates synergistic inhibition of NF-  $\kappa$ B translocation from cytoplasm to nucleus (140).

This is the first study reporting the enhanced action in modulation of pro-inflammatory genes and proteins by natural complementarily flavonoid mixtures with practical relevance to diet, which demonstrates significant structural complementarity of the natural flavonoids with 3-deoxy and 3-hydroxy groups against inflammation. Furthermore, strong dependence of sorghum-cowpea ratios implies their active compounds act via different mechanisms. The main flavonoid compounds identified in sorghum and cowpea; apigenin and quercetin have been separately shown to inhibit NF-  $\kappa$ B activation and its related downstream genes in human cell models through multiple mechanisms (23, 68, 72, 149). Therefore, this study suggests that the co-administration of structurally complementary natural flavonoids present in sorghum and cowpea, targeting different signal mechanisms has the potential to increase anti-inflammatory efficiency. This implies lower doses of each can be used, which is easily achievable through habitual diet.

#### *Effects of White Sorghum-White Cowpea Mixtures on Pro-Inflammatory Cytokine Gene and Protein Expression*

At highest concentration tested (1.0  $\mu$ g/mL), sorghum-cowpea extract combinations showed significantly ( $P < 0.05$ ) higher down regulation of pro-inflammatory cytokines

(TNF- $\alpha$ , IL-6, IL-8) mRNA expression than the additive effect of individual extracts (Fig. 16-22). Sorghum-cowpea mixtures in different ratios (at similar concentrations), revealed significantly ( $P<0.05$ ) different down regulation in all pro-inflammatory cytokines (TNF- $\alpha$ , IL-6, IL-8) mRNA (Fig.16-22). Major findings for the different markers are summarized below:

**TNF- $\alpha$  :** The minimum effective dose that showed significant down regulation of LPS induced TNF- $\alpha$  gene expression for additive effect was 1.0  $\mu\text{g/mL}$ ; whereas the sorghum-cowpea mixtures significantly ( $P<0.05$ ) inhibited TNF- $\alpha$  gene expression at 0.1  $\mu\text{g/mL}$  (Fig.. 16, 17). The sorghum-cowpea ratios significantly ( $P<0.05$ ) affected the magnitude of synergy observed in TNF- $\alpha$  gene regulation. For example: At 0.1  $\mu\text{g/mL}$ , combination in 1:3 ratio (43% down-regulation) was significantly ( $P<0.05$ ) more effective at inhibiting TNF- $\alpha$  gene expression compared to the combination in 1:1 ratio (14%) (Fig. 16, 17).

**IL-6:** The minimum effective dose that showed significant attenuation of LPS induced IL-6 gene expression for additive effect of individual extracts was 0.1  $\mu\text{g/mL}$ ; while the sorghum-cowpea mixtures significantly ( $P<0.05$ ) reduced IL-6 gene expression at 0.05  $\mu\text{g/mL}$  (Fig. 19, 20). The magnitude of synergistic reduction of IL-6 gene expression was also significantly ( $P<0.05$ ) dependent on the relative proportions of sorghum-cowpea mixtures. As shown in Fig. 20, at 0.05  $\mu\text{g/mL}$  concentration, mixture with 1:1 ratio showed stronger inhibitory effect (90%) on IL-6 gene expression than the mixture with (3:1) ratio (35%).

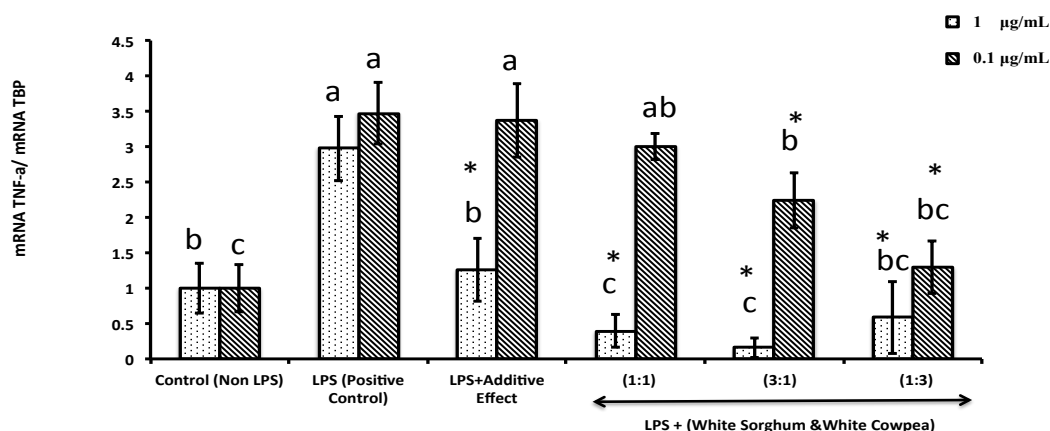
**IL-8:** The minimum effective dose that revealed significant reduction of LPS induced IL-8 gene expression for additive effect of was 0.05  $\mu\text{g/mL}$ ; whereas the sorghum-cowpea mixtures significantly ( $P<0.05$ ) inhibited IL-8 gene expression at 0.01  $\mu\text{g/mL}$  (Fig. 22, 23).



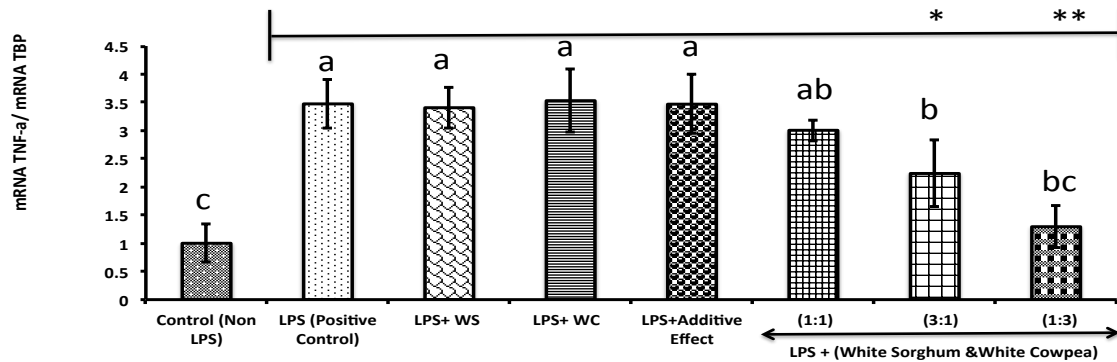
The ratios of sorghum-cowpea mixtures also significantly ( $P<0.05$ ) influence the magnitude of synergy for IL-8 gene expression reduction. For instance: The sorghum-cowpea mixture in 1:1 ratio exhibited higher reduction (78%) than the mixture in 3:1 ratio (57%) at 0.01  $\mu\text{g/mL}$  (Fig. 23). The magnitude of synergistic effect and the minimum effective doses were different for the pro-inflammatory cytokines tested in this study. These differences could be due to the complexity of the interactions between crude mixtures components on inflammation response. Active components present in the sorghum-cowpea crude mixtures could inhibit pro-inflammatory cytokines via different mechanisms. The concentration of active compounds may also influence the nature of these interactions.

In order to test whether the down-regulation of the mRNA expression of pro-inflammatory cytokines by sorghum-cowpea mixtures was correlated to the mitigation of protein levels, we measured pro-inflammatory cytokines (TNF- $\alpha$ , IL-6, IL-8) protein expression by multiple beads at concentrations (0.1, 0.05, 0.01  $\mu\text{g/mL}$ ). The protein expressions of TNF- $\alpha$ , IL-6, and IL-8 were also synergistically decreased by sorghum-cowpea mixtures compared to the additive effect (Fig. 18, 21, 24), which agree with gene expression results. Corresponding to mRNA results, the ratios of sorghum-cowpea mixtures significantly ( $P<0.05$ ) impacted the magnitude of synergistic inhibitory effect on pro-inflammatory cytokines protein expression (Fig. 18, 21, 24). The combination in 1:3 ratio were more effective in reducing TNF- $\alpha$  protein expression compared to the combination in 1:1 ratio (Fig. 18). Co-treatment of sorghum-cowpea mixtures in 3:1 ratio had stronger effect (42%) against IL-8 expression than the mixture in 1:3 ratio

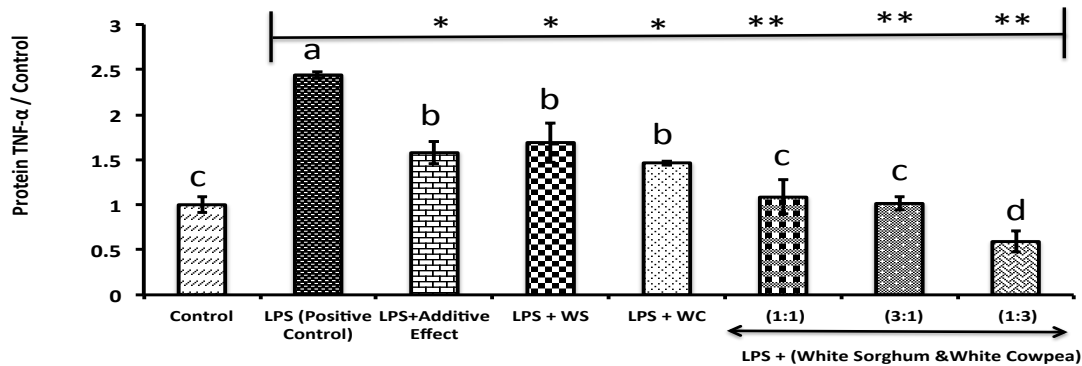
(15%) (Fig. 24).



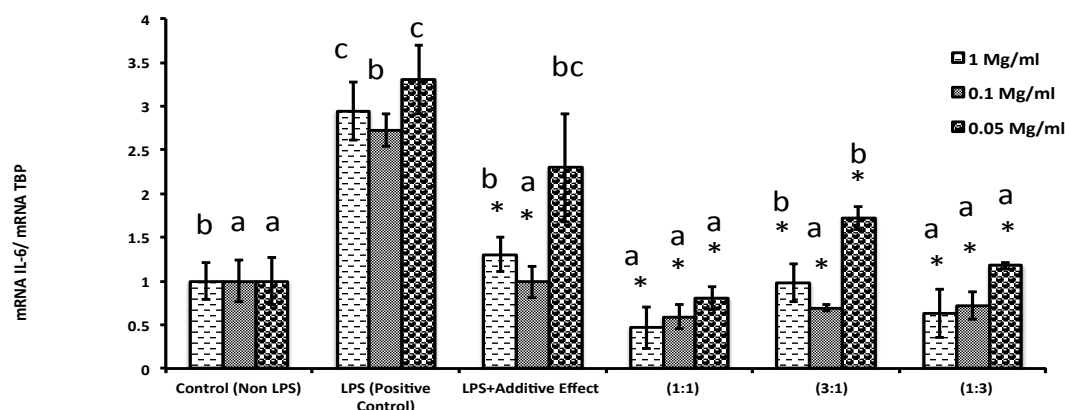
**Figure 16:** Effect of extracts of sorghum and cowpea mixtures on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.1-1.0  $\mu\text{g/mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



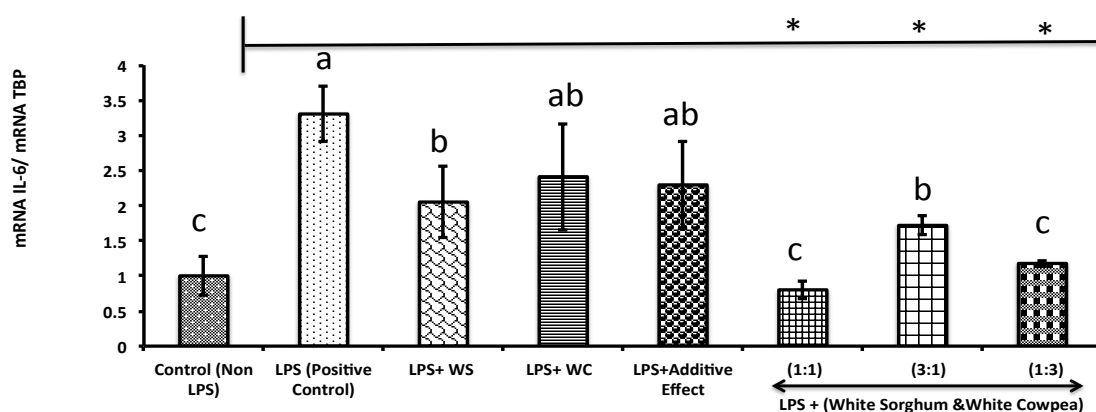
**Figure 17:** Effect of extracts of sorghum and cowpea mixtures on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.1  $\mu\text{g/mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



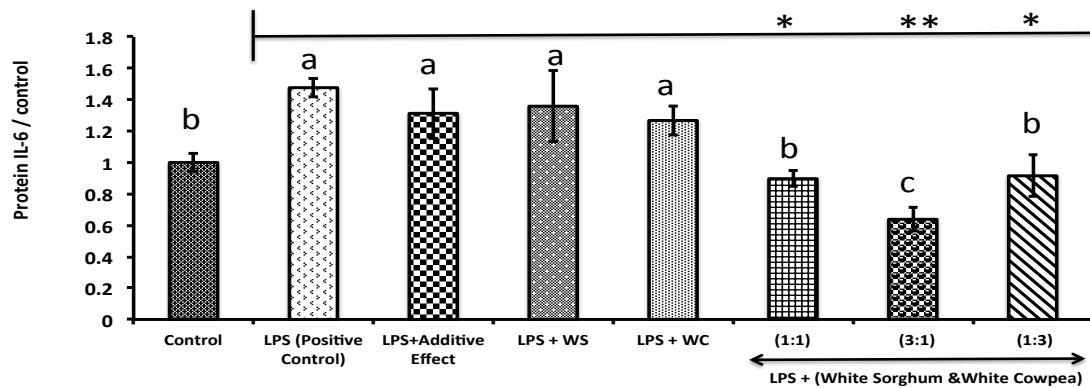
**Figure 18:** Effect of extracts of sorghum and cowpea mixtures on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) protein expression in CCD-18Co cells. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu\text{g/mL}$ ) of sorghum and cowpea combinations, and challenged with LPS (10  $\mu\text{g/mL}$ ) for 24 h. Protein expressions were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



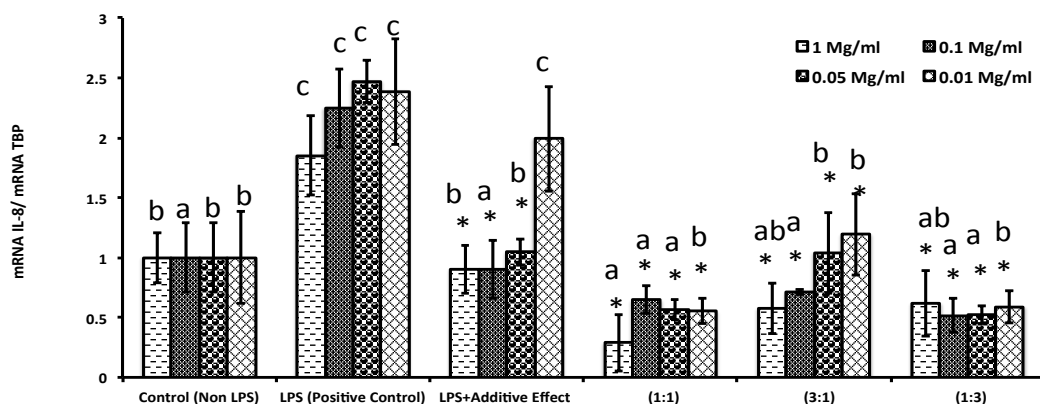
**Figure 19:** Effect of extracts of sorghum and cowpea mixtures on interleukin-6 (IL-6) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.05-1.0  $\mu\text{g}/\text{mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



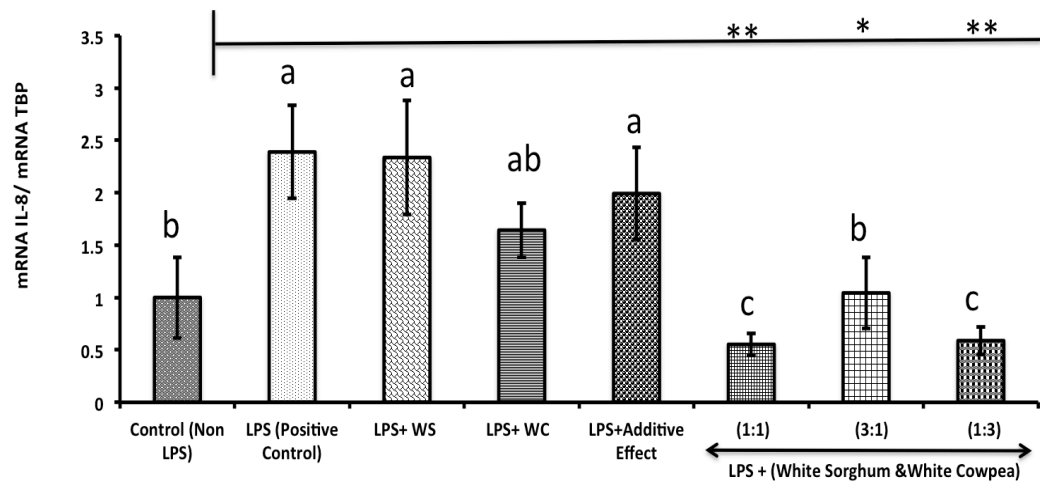
**Figure 20:** Effect of extracts of sorghum and cowpea mixtures on interleukin-6 (IL-6) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.05  $\mu\text{g}/\text{mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



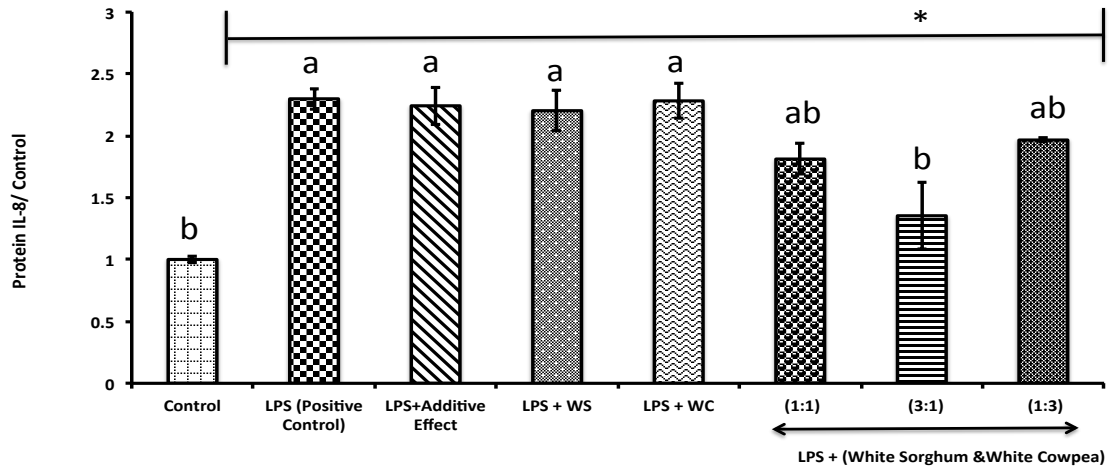
**Figure 21:** Effect of extracts of sorghum and cowpea mixtures on interleukin-6 (IL-6) protein expression in CCD-18Co cells. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.05  $\mu\text{g}/\text{ml}$ ) of sorghum and cowpea combinations, and challenged with LPS (10  $\mu\text{g}/\text{ml}$ ) for 24 h. Protein expressions were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure 22:** Effect of extracts of sorghum and cowpea mixtures on interleukin-8 (IL-8) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.01-1.0  $\mu\text{g}/\text{mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure 23:** Effect of extracts of sorghum and cowpea mixtures on interleukin-8 (IL-8) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at 0.01  $\mu\text{g/mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure 24:** Effect of extracts of sorghum and cowpea mixtures on interleukin-8 (IL-8) protein expression in CCD-18Co cells. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.01  $\mu\text{g/ml}$ ) of sorghum and cowpea combinations, and challenged with LPS (10  $\mu\text{g/mL}$ ) for 24 h. Protein expressions were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

Inflammatory cytokines such as TNF- $\alpha$  produce ROS that can be an important signal for other biological effects in cells such as proliferation and programmed cell death (90). IL-8 are chemokines, which causes secretion of ROS by promoting phagocytosis. Furthermore, IL-8 are secreted by endothelial cells under oxidative stress (72, 90). Several studies have implicated ROS in the etiology of several chronic disorders such as colon cancer and IBD (72, 90). Interlukin-6 (IL-6) have been linked to tumorigenesis suggesting that inflammation is related with cancer development.

A few studies have demonstrated synergistic down-regulation of pro-inflammatory cytokines by phytochemical mixtures (22, 48, 97, 116, 140), but these studies are still limited to purified polyphenol mixtures at higher concentrations compared to our study. For example: Park *et al.*, 2011 have reported the co-treatment of luteolin and chironic acid inhibited TNF- $\alpha$  and IL-1 $\beta$  protein expression more strongly (50%) than the individual treatments at the minimum effective dose tested (12  $\mu$ M) in LPS induced RAW 264.7 cells, but no significant differences was observed between combinations in 1:1, 1:2 and 1:4 ratios (97). Another study reported that the co-incubation of carotenoids and polyphenols (including 1  $\mu$ M lycopene with 1  $\mu$ M lutein, 2  $\mu$ M carnosic acid, and 2  $\mu$ M  $\beta$ -carotene individually) resulted in stronger inhibition of TNF- $\alpha$  by almost 30%, 45% and 30% respectively. These results suggested that carotenoids and polyphenols act through disparate mechanisms (48). This is in agreement with a recent animal study, which showed that, the individually mixtures of  $\beta$ -carotene (10 mg/kg) with low doses (50 mg/kg) and high doses (100 mg/kg) of quercetin synergistically decreased the levels of TNF- $\alpha$  and IL-6 in plasma. The dose effect of quercetin was significant in reducing the plasma level of IL-6; the mixture with 1:10 ( $\beta$ -carotene: quercetin) ratio was more effective than the mixture with 1:5; whereas for TNF- $\alpha$  the dose effect of quercetin was not significant (140).

Our study is the first study to report synergistic down-regulation of pro-inflammatory cytokines gene and protein expression by natural complementary flavonoid mixtures as found in foods with practical relevance to diet. Consistent synergistic anti-inflammatory effects of flavonoids present in white sorghum-white cowpea mixtures enhance their



bioactive properties. Since their effective concentrations are likely achievable through diet, this study provides information that may be used to optimize cereal-legume combinations that could efficiently contribute to chronic disease prevention.

Apigenin as the most abundant anti-inflammatory flavonoid in white sorghum ( $5.5 \pm 0.12$  mg flavones/g extract) is known to inhibit NF- $\kappa$ B activation and its related downstream genes via multiple mechanisms. For example: Apigenin inhibited LPS-induced IL-1 $\beta$  production by inhibiting caspase-1 activation and also prevented LPS-induced IL-6 production by reducing the mRNA stability via inhibiting ERK1/2 activation (149). In another study, apigenin (10  $\mu$ M) suppressed LPS induced COX-2 expression in RAW 264.7 cells by inhibiting arachidonic acid (AA) release causing suppression of PGs synthesis (72). Quercetin derivatives, the most dominant anti-inflammatory flavonoid in white cowpea ( $7.3 \pm 2.7$  mg flavonols/g extract) was also revealed to down regulate the activating of NF- $\kappa$ B pathway in a dose dependent manner by inhibiting the phosphorylation of I $\kappa$ B- $\alpha$  protein induced by LPS in macrophages which resulted in reduction in TNF- $\alpha$  and IL-1 $\beta$  secretion by almost 40% (23). Quercetin was also reported to reduce the gene expression of specific factors implicated in local vascular inflammation including IL-1R, Ccl8, IKK, and STAT3 (68). Hence, the combination of these compounds and their derivatives from sorghum and cowpea extracts likely acted via different mechanisms to synergistically enhance activity. Therefore, apigenin and quercetin were further tested in this study to determine the potential contribution of the flavones and flavonols in the synergistic effect of sorghum-cowpea mixtures against inflammation.

### *Effects of Apigenin-Quercetin Mixtures on NF- $\kappa$ B and Pro-Inflammatory Cytokines Gene and Protein Expression*

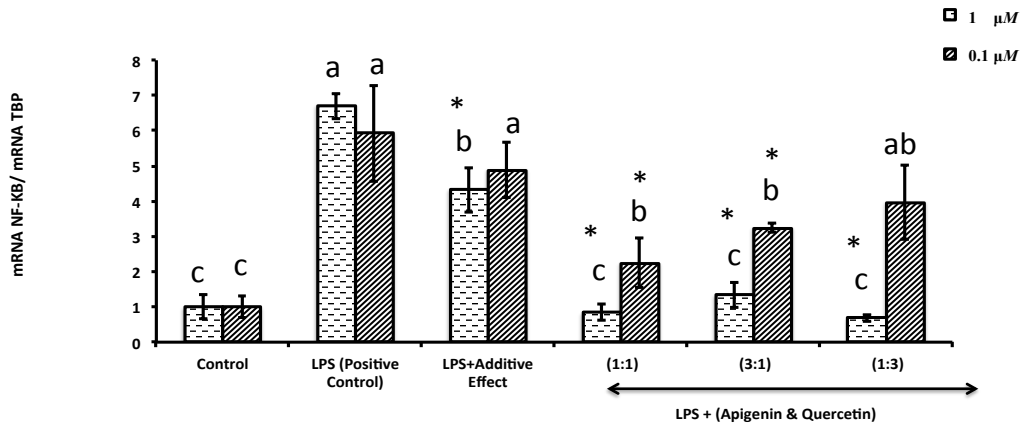
The apigenin-quercetin mixtures were more potent in down regulation of NF- $\kappa$ B and all pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-8 ) gene expression than the additive effect of individual extracts, which is consistent with results on the synergistic anti-inflammatory effect of sorghum-cowpea mixtures (Fig. 25, 28, 31, 34). The minimum effective dose that revealed significant inhibition of LPS induced NF- $\kappa$ B and pro-inflammatory cytokines gene expression for additive effect of individual extracts was (1  $\mu$ M); whereas the apigenin-quercetin mixtures significantly ( $P < 0.05$ ) down regulated NF- $\kappa$ B gene expression at 0.1  $\mu$ M (Fig. 25, 28, 31, 34). These results support the hypothesis that flavone/flavonol mixtures can synergistically regulate inflammation response.

Corresponding to the sorghum-cowpea mixtures results, apigenin-quercetin combinations in different ratios (1:1, 1:3, 3:1) (at similar concentrations), displayed significantly ( $P < 0.05$ ) different inhibitory effect in NF- $\kappa$ B and pro-inflammatory cytokines mRNA (Fig. 25, 28, 31, 34). For instance: The combination in 1:3 ratio was more effective (63%) in inhibiting NF- $\kappa$ B gene expression compared to the combination in 3:1 ratio (44%) at 0.1  $\mu$ M (Fig.26). As shown in Fig.32, at 0.1  $\mu$ M concentration, mixture with (1:1) ratio showed significantly ( $P < 0.05$ ) stronger mitigation (92%) in IL-6 gene expression than the mixture with (1:3) ratio (25%). The combinations in 1:1 and 1:3 ratios were more effective in reducing IL-8 expression compared to the combination in 3:1 ratio (Fig. 35). Such strong dependency on relative proportion is in agreement with previous results. Jonker *et al.*, 2005 (63) demonstrated that in order to correctly

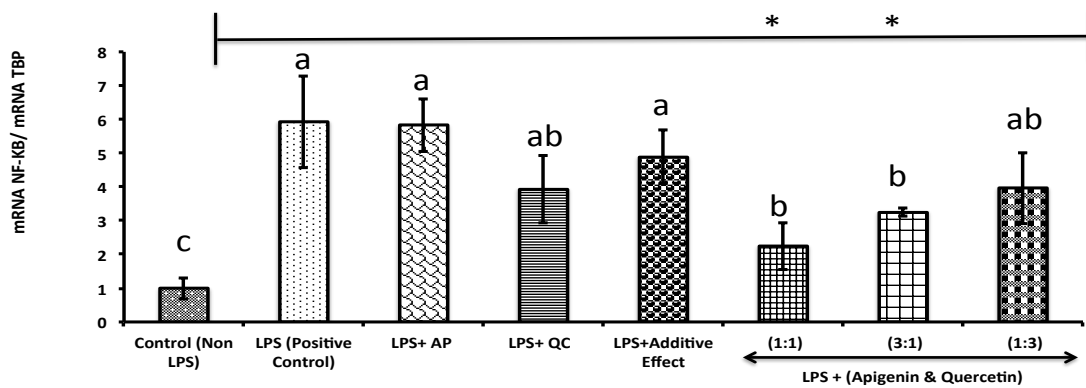
analyze the synergism/antagonism of mixture effects, more complex response patterns such as dose ratio or dose level deviation patterns should be addressed.

Results showed that reduction of NF- $\kappa$ B and pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-8) mRNA expression by apigenin-quercetin mixtures was correlated with the mitigation of protein levels (Fig. 27, 30, 33, 36). Apigenin-quercetin mixtures exhibited synergistic reduction in NF- $\kappa$ B and pro-inflammatory cytokines protein expression at 0.1  $\mu$ M, which was the minimum effective dose that revealed synergistic effect in NF- $\kappa$ B gene expression results (Fig. 27). The western blot analysis showed stronger reduction in p-NF- $\kappa$ B/NF- $\kappa$ B ratio (58%) than the additive effect (37%) in the same treatment (Fig. 27). The results from multiple bead analysis showed that co-treatment of apigenin-quercetin mixture in 1:1 ratio revealed greater inhibition in TNF- $\alpha$  protein expression (78%) compared to the additive effect of individuals (27%) (Fig. 30), which support the mRNA analysis results for the same treatment.

Apigenin-quercetin mixtures in different ratios also revealed significantly ( $P < 0.05$ ) different down regulation in NF- $\kappa$ B and pro-inflammatory cytokines in protein expression as well (Fig. 27, 30, 33, 36). For example: the combination in 1:3 ratio (58%) was more effective in reducing p-NF- $\kappa$ B/NF- $\kappa$ B ratio compared to the combination in 1:1 ratio (10%) (Fig. 27). Co-treatment of apigenin-quercetin mixtures in 1:3 ratio had stronger anti-inflammatory effects (58%) against IL-8 expression than the mixture in 1:1 ratio (25%) (Fig. 36).

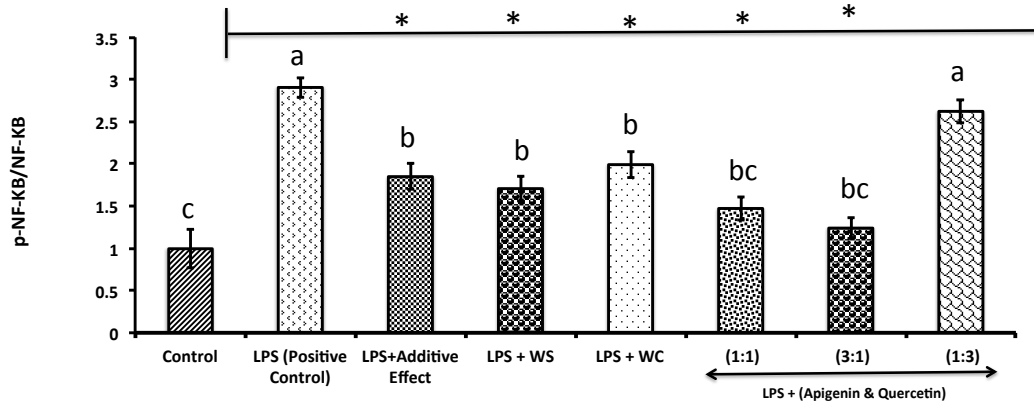


**Figure 25:** Effect of apigenin and quercetin mixtures on nuclear factor- $\kappa$ B (NF- $\kappa$ B) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1-1.0  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

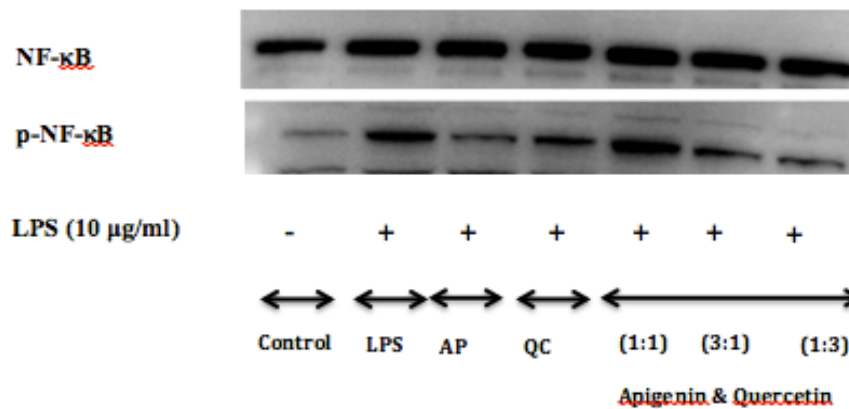


**Figure 26:** Effect of apigenin and quercetin mixtures on nuclear factor- $\kappa$ B (NF- $\kappa$ B) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

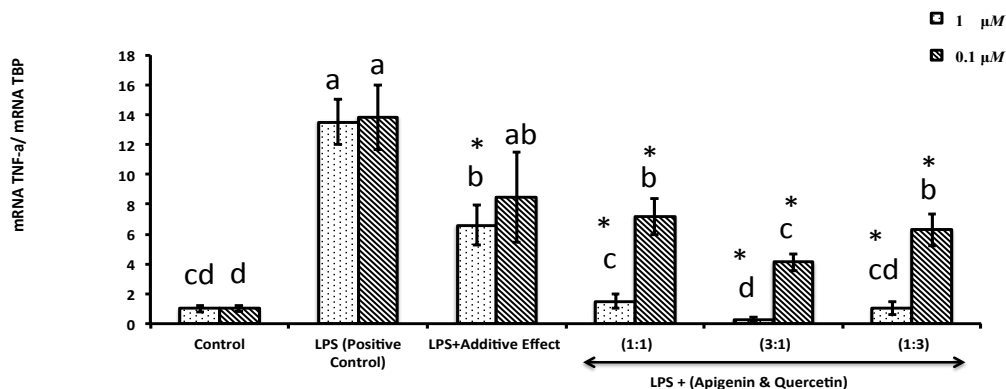
A



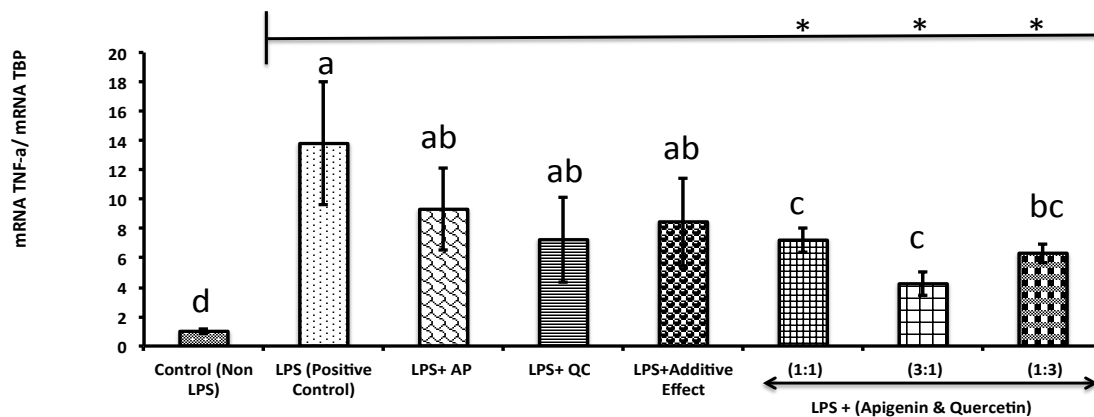
B



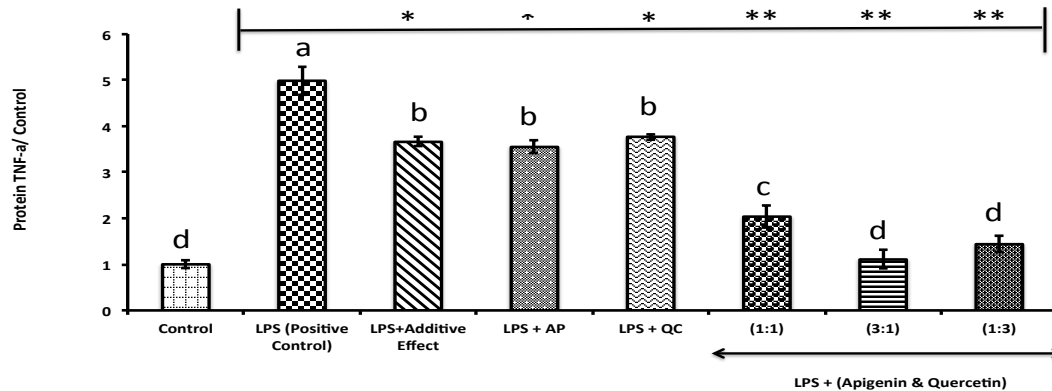
**Figure 27:** Effect of apigenin and quercetin mixtures on nuclear factor-  $\kappa$ B (NF- $\kappa$ B) protein expression in CCD-18Co cells. Ratio of CCD-18Co phospho-Nuclear Factor- $\kappa$ B / Nuclear Factor- $\kappa$ B ( $\rho$ -NF- $\kappa$ B/NF- $\kappa$ B) protein expression. Cells were treated with different (0.1  $\mu$ M) apigenin and quercetin combinations for 24 h, then challenged with LPS for 24 h. Protein expression was analyzed by Western Blot, normalized with untreated control cells (A). Western blot analysis of  $\rho$ -NF- $\kappa$ B/NF- $\kappa$ B (B). Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



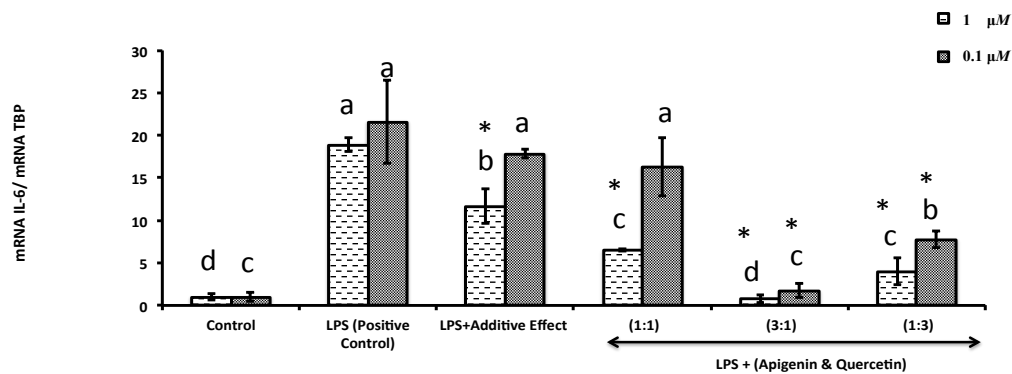
**Figure 28:** Effect of apigenin and quercetin mixtures on tumor necrosis factor- $\alpha$  (TNF-  $\alpha$ ) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1-1.0  $\mu M$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



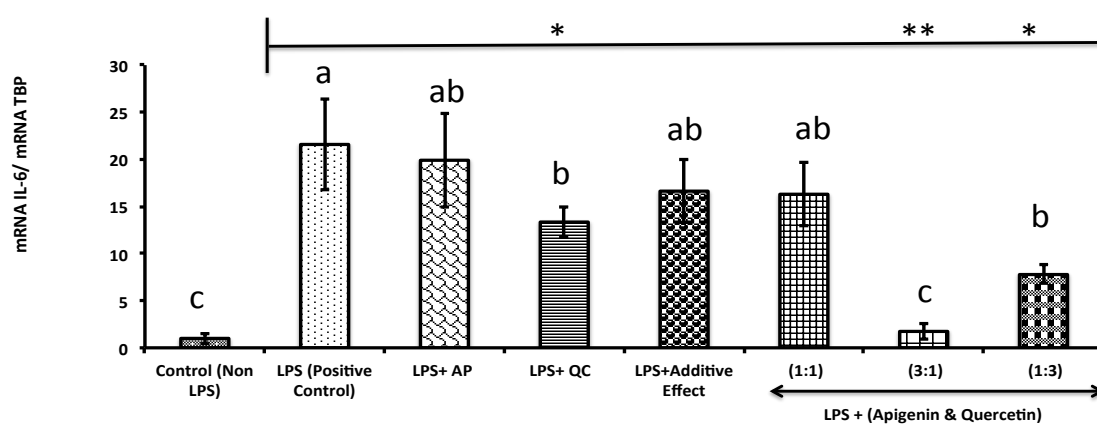
**Figure 29:** Effect of apigenin and quercetin mixtures on tumor necrosis factor- $\alpha$  (TNF-  $\alpha$ ) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1  $\mu M$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



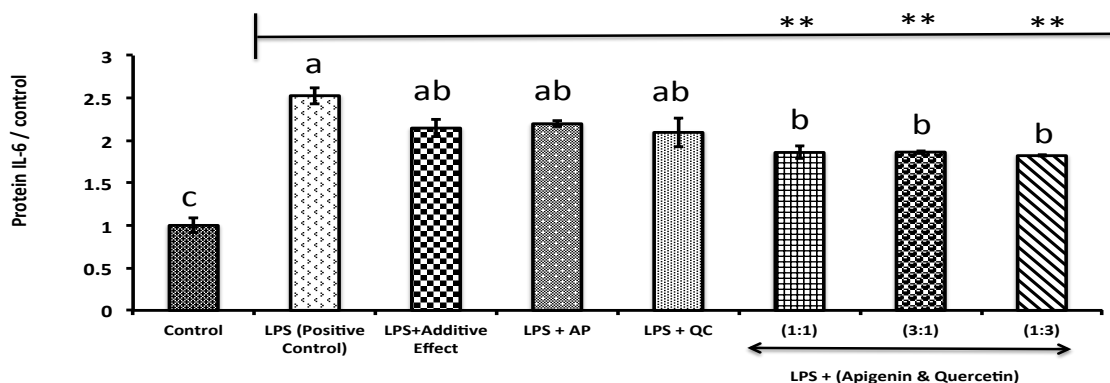
**Figure 30:** Effect of apigenin and quercetin mixtures on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) protein expression in CCD-18Co cells. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ M) of apigenin and quercetin combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h. Protein expressions were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure 31:** Effect of apigenin and quercetin mixtures on interleukin-6 (IL-6) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1-1.0  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

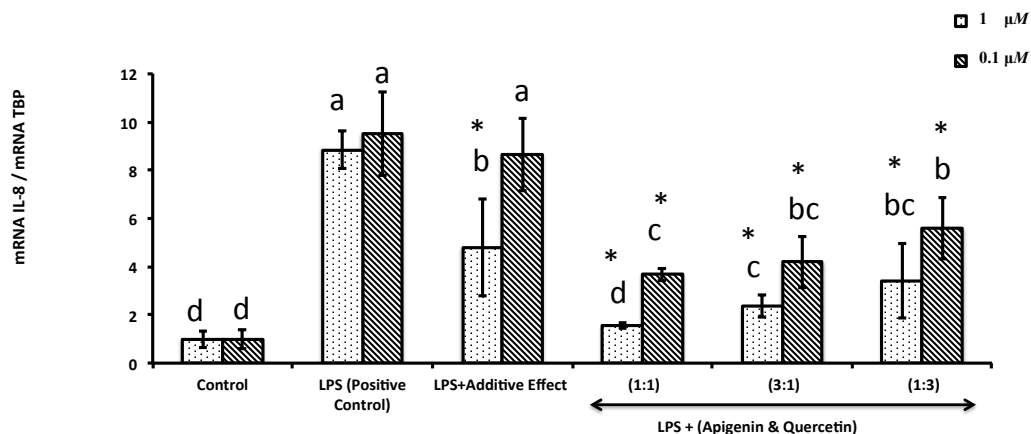


**Figure 32:** Effect of apigenin and quercetin mixtures on interleukin-6 (IL-6) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

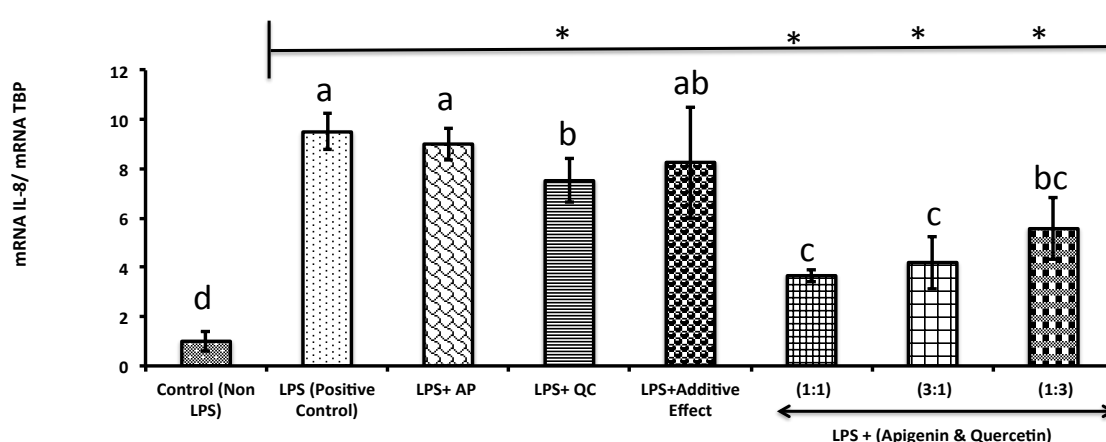


**Figure 33:** Effect of apigenin and quercetin mixtures on interleukin-6 (IL-6) protein expression in CCD-18Co cells. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ M) of apigenin and quercetin combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h. Protein expressions were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

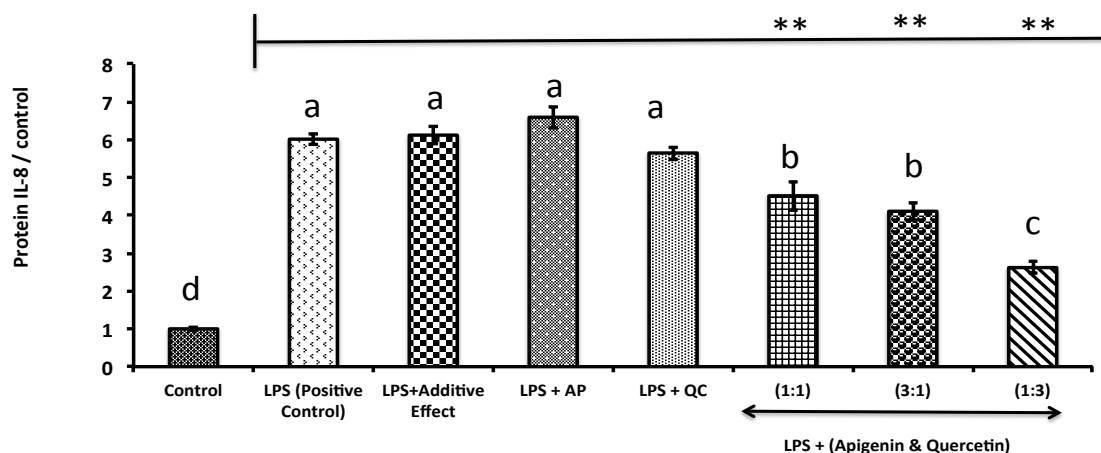




**Figure 34:** Effect of apigenin and quercetin mixtures on interleukin-8 (IL-8) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1-1.0  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure 35:** Effect of apigenin and quercetin mixtures on interleukin-8 (IL-8) gene expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at 0.1  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure 36:** Effect of apigenin and quercetin mixtures on interleukin-8 (IL-8) protein expression in CCD-18Co cells. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ M) of apigenin and quercetin combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h. Protein expressions were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

As previously discussed, Park *et al.*, 2011 reported luteolin as the main inflammation response suppressor in luteolin-chicoric acid mixture and that chicoric acid just strengthened luteolin's effect (97). Hazewindus *et al.*, 2012 reported that in lycopene, ascorbic acid and  $\alpha$ -tocopherol combination, lycopene mitigated inflammation response; whereas ascorbic acid and  $\alpha$ -tocopherol efficiently inhibited lipid peroxidation. Both activities complemented each other, which resulted in synergistic reduction of inflammation process (50).

In our study, small non-significant difference was observed between individual apigenin and quercetin in reducing expression of NF- $\kappa$ B and pro-inflammatory

cytokines, whereas significant differences were apparent among different ratios of mixtures, indicating that both apigenin and quercetin separately reduced inflammatory response through different mechanisms, which is in agreement with previous studies (9, 22, 26, 53). Apigenin, as an important dietary flavone with strong anti-inflammatory effect, has been shown to significantly reduce IL-6, IL-1 $\beta$  and TNF- $\alpha$  mRNA levels in LPS-activated macrophages and mouse model (149). The C2-C3 double bond along with 4-oxo functional group of the heterocyclic ring is essential to the high anti-inflammatory effect (149). Furthermore, the hydroxylations at positions 5, 7 are very important for strong anti-inflammatory effects. Quercetin as an important dietary flavonol reduced cytokine and inducible nitric oxide synthase expression through inhibition of NF- $\kappa$ B pathway (both *in-vitro* and *in-vivo*) (23). Conjugation between the 3-hydroxyl group in C ring with the 4-oxo (carbonyl group) may boost the effect of quercetin against inflammation (23).

Thus, our results suggest that co-administration of structurally complementary flavonoids (apigenin-quercetin) mixtures, targeting different signaling mechanisms has the potential to increase anti-inflammatory efficiency. The evidence supports our hypothesis that flavone/flavonol mixtures are responsible for synergistic down regulation of inflammation response by sorghum-cowpea mixtures.

Although consistent synergy in regulating inflammation process was observed in both sorghum-cowpea and apigenin-quercetin results, indicating the likely dominant contribution of flavones and flavonols in synergistic effects, the minimum effective concentrations that showed synergistic effect in sorghum-cowpea extracts ( $\approx$  0.12-1.2 ng flavonoid/mL) mixtures had much (200 times) lower than the active levels of apigenin-quercetin mixtures ( $\approx$  28.5-285 ng

flavonoids/mL). Previous studies had demonstrated that using isolated/purified compounds may not be a good predictive model for establishing magnitude the bioactivity of flavonoids in foods (*143*). The potential activity of a given compound in a complex natural combination found in most foods might be higher than can predicted by applying purified compounds. A possible explanation is that in a natural mixture (sorghum-cowpea mixture) in this case, small quantities of diverse anti-inflammatory compounds may favorably interact to enhance inflammation modulation in complex ways.

Despite relatively high dietary intake of flavonoids, their effective bioavailability (and by extension bioactivity) is relatively poor. This can be attributed to their metabolism by phase II enzymes in intestinal epithelial cells and/or increased efflux from intestinal cells back to the lumen (apical side), mediated by ATP binding cassette (ABC) transport proteins (*18, 19, 76*). Flavonoids and their metabolites, depending on their structure, might act as inducers or inhibitors of ABC transporters (*20, 34, 82*). Most anti-inflammatory flavonoids including apigenin (dominant flavonoid in white sorghum) and quercetin and its glycosides (major flavonoids in white cowpea) generally act as inhibitors of transporter proteins (*82, 133, 147*). This suggests that the synergistic anti-inflammatory effect we observed for the flavonoids present in sorghum-cowpea mixtures in non-malignant CCD-18Co cells may be partly dependent on their interaction with the ABC transporters. Moreover, literature demonstrated that co-administration of hesperitin with quercetin significantly diminished apical efflux and enhances excretion on basolateral side of Caco2 cells, which resulted in improving bioavailability of hesperotin (*19*). The synergistic anti-inflammatory effect of sorghum-cowpea flavonoids

combinations at low concentrations in non-malignant CCD-18Co cells is thus possibly contributed by complex interactions involving different mechanisms that may involve the ABC transporters. We investigate this hypothesis in Chapter 5.

## **Conclusions**

The White sorghum-white cowpea combinations were able to synergistically attenuate the process of inflammation in non-cancer LPS-induced CCD-18Co cells by inhibiting the activation of NF- $\kappa$ B and related downstream targets such as IL-6, IL-8 and TNF- $\alpha$  at both mRNA and protein levels. The combination of apigenin and quercetin significantly enhanced their action in reducing inflammation response by inhibiting the same biomarkers expression. The enhanced action in apigenin-quercetin mixtures was adequate to explain the contribution of flavones and flavonols in the synergistic action of sorghum-cowpea mixtures against inflammation. The magnitude of synergy was strongly dependent on the relative proportions in both sorghum-cowpea and apigenin-quercetin mixtures, which suggests different mechanisms for the flavones and flavonols. Thus strategic cereal and legume combinations might be formulated for optimized benefits in chronic disease prevention.

This research provides a foundation for future *in vivo* studies linked to intestinal inflammation. Results from *in-vitro* study provide evidence of the potential enhanced action of sorghum-cowpea flavonoid mixtures in modulating the level of inflammatory markers linked to colon inflammation. The possible underlying mechanism that contributes to the enhanced anti-inflammatory action of sorghum-cowpea mixtures need

further investigation. These results suggest that the combinations of white sorghum and white cowpea could be considered as a source of dietary polyphenols capable of inhibit the detrimental effects of inflammation in colon cells at levels relevant to diet. This has a lot of immediate relevance to the health of vulnerable populations in low-income regions where most sorghum and cowpea are produced and consumed.

## **CHAPTER V**

### **THE EFFECT OF SORGHUM AND COWPEA FLAVONOID MIXTURES ON ATP BINDING CASSETTE (ABC) TRANSPORTER PROTEINS USING CACO- 2 CELL MODEL**

#### **Introduction**

Epidemiological studies have established an association between whole grain and grain legume intake and reduced the risk of CVD, cancer and diabetes, which may be related to their polyphenol compositions and dietary fiber, among others (71, 79, 124). Sorghum and cowpea are both well adapted to semi arid regions and perform better than other cereals and legumes under environmental stresses (e.g. high heat and low moisture climate), thus they are produced and consumed together (e.g. in west Africa) (8, 85). Recent evidences demonstrate that sorghum and cowpea contain a wide variety of distinctly different flavonoids with different structural profiles that may provide complementary bioactive properties (8, 92, 93). In Chapter 4 we showed strong synergistic interaction in white sorghum -white cowpea flavonoids (flavones and flavonols) combinations against chronic inflammation in non-malignant myofibroblast CCD-18Co cells. Bioactive compounds such as flavonoids are ingested in combinations in the human diet, understanding the mechanisms by which they may synergistically contribute to chronic disease prevention is important in order to maximize their impact on human health.

Despite high dietary intake of flavonoids, their effective bioavailability (and by extension bioactivity) is relatively poor (18, 19, 76). This can be partly attributed to their metabolism by phase II enzymes and/or increased efflux from intestinal cells back to the lumen (apical side), mediated by ATP binding cassette (ABC) transport proteins (18, 19, 76). Intestinal ABC transporters reported to be involved in flavonoid transportation, including breast cancer resistance protein (BCRP/ ABCG2), multidrug resistance protein (MRPs/ABCCs) and P-glycoprotein (*P*-gp/MDR1/ABCB1) are localized in the apical membrane (18, 19, 76). Several studies have shown that, depending on their structure; flavonoids and their metabolites might act as inducers or inhibitors of ABC transporters (BCRP, *P*-gp and MRP2) (20, 34, 82). Quercetin, kaempferol, hesperitin, and naringenin inhibited the *P*-gp in MBEC4 cell model (cultured mouse brain capillary endothelial cells), which resulted in increased vincristine (chemotherapy medication used to treat a number of types of cancer by stopping cells from dividing properly) uptake (81). Moreover, another study revealed that depending on the treatment concentration and exposure time, chrysin, a flavone, seemed to play a dual role in regulating MRP2 in Caco-2 cells, which showed both inhibition and enhancement in expression of MRP2 (117, 120).

Until now, very few studies have assessed synergistic effects of flavonoid combinations relevant to complementary foods in modulating transporter proteins (4, 18, 19, 147). Several authors have reported synergistic effect of isolated flavonoid combinations. For example: Brand *et al* 2008, demonstrated that co-administration of hesperitin with quercetin significantly diminished apical efflux and enhance its excretion



on basolateral side of Caco-2 cells. Furthermore, co-administration of 5,7-dimethoxyflavone (5,7-DMF) with multiple other flavones (7,8-benzoflavone, 5, 6, 7-trimethoxyflavone and 8-methylflavone) in mouse tissue showed higher inhibition of BCRP, which resulted in increasing area under curve (AUC) values of mitoxantrone in different mouse tissues (4).

Most anti-inflammatory flavonoids including apigenin (dominant flavonoid in white sorghum) and quercetin and its glycosides (major flavonoids in white cowpea) generally act as inhibitors of transporter proteins (82, 113, 146, 148). Based on evidence on relationship between flavonoids and transporter proteins, we hypothesized that structurally different flavonoid classes targeting different signaling mechanisms could achieve a greater inhibitory effect. In this context, we hypothesized that synergistic effect in modulating the transporter protein expression by sorghum-cowpea flavonoid mixtures could be a possible mechanism for the interactive effect of sorghum-cowpea flavonoids combinations we observed against inflammation in non-malignant colon cells. In this study, we demonstrate synergistic interaction of sorghum-cowpea flavonoids against protein transporters expression using Caco2 cell monolayers.

## **Materials and Methods**

### *Plant Material*

The white cowpea variety (09FCV-E CB27), containing mainly flavonols (quercetin glycosides), was gathered in experimental station in College Station, Texas in July 2011. White sorghum variety (ATX635×RTX436), which contains flavones, (mainly apigenin and luteolin) as the dominant phenols, was provided by Dr W. L. Rooney in Department of Soil and Crop Sciences, Texas AandM University. The sorghum grains were harvested in 2011 at College station, TX and stored in -20 °C until use. Prior to use, foreign materials, broken and damaged seeds were separated.

### *Sample Preparation*

Whole kernel sorghums and dry seed cowpeas were ground using a coffee grinder (Cuisinart, Model DCG-20N series) to pass through a 60-mesh sieve. Ground sorghum and cowpea varieties were extracted with 70% aqueous acetone, which detailed in Chapter 3, roto-evaporated, then the extracts were freeze-dried and stored at -80°C until use.

### *Chemicals and Reagents*

Pure standards used in this investigation were as follows: Apigenin was from Indofine (Indofine Chemical Company, Inc., Hillsborough, NJ); quercetin was from

Sigma-Aldrich Chemicals, St. Louis, MO. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The forward and reverse pair primers (BCRP (ABCG2), MDP1 (ABCB1), MRP2 (ABCC2), MRP3 (ABCC3) and Villin 1 (VIL 1)) were obtained from Integrated DNA Technologies, Inc. (San Diego, CA). Bradford reagent was obtained from BioRad (Hercules, CA), Antibodies against BCRP (ABCG2), MDP1 (ABCB1), MRP2 (ABCC2), MRP3 (ABCC3) were obtained from Cell Signaling Technology (Beverly, MA). Cyclosporin A, a broad-spectrum transporter protein inhibitor, was also purchased from Cell Signaling Technology (Beverly, MA).

### *Cell Culture*

Caco-2 colon adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and incubated at 37 °C and 5% CO<sub>2</sub> in growth media, consisted of EMEM 20 % FBS, 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO). Caco-2 cells are a good model for the absorptive and defensive properties of the intestinal mucosa. These cells as a model of intestinal barrier are able to differentiate spontaneously in culture forming monolayers of cells, which express morphological and functional characteristics of the mature enterocytes (114).

### *Gene Expression Experiments*

Cells were seeded in 12 well plate at a population density of 250,00 cells/well, incubated at 37°C/ 5% CO<sub>2</sub>. Seeded cells were grown and differentiated to confluent monolayers for 15-23 days. In order to monitor the confluence and integrity of monolayer cells, transepithelial electrical resistance (TEER) values were measured with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL). TEER values were measured to insure the monolayers integrity. Monolayers with appropriate TEER values (>450  $\Omega$  cm<sup>2</sup> at the beginning and >250  $\Omega$  cm<sup>2</sup> at the end) were used for analysis. Non-confluent monolayers with lower TEER values were discarded. Treatments pre-dissolved in DMSO were diluted and normalized in culture medium to reach the appropriate concentrations (0.01-1.0  $\mu$ g/mL) (< 0.2% DMSO). Cyclosporin A, a broad-spectrum inhibitor reported to modulate most of the transporter proteins including BCRP, *P*-gp and MRPs, was used in this study as a positive control at 10  $\mu$ M. This concentration was reported to inhibit mitoxantrone efflux in *P*-gp, MRPs, and BCRP overexpressing cell lines (99, 106).

The cells were subsequently treated with white sorghum and white cowpea extract combinations at different ratios (1:1, 1:3, 3:1) (at 0.01-1.0  $\mu$ g/mL concentrations, which correspond to the concentrations used in inflammatory assays in Chapter 4) and cyclosporine A (10  $\mu$ M) for 6 h. Messenger RNA (mRNA) was then extracted from the lysated cells and analyzed. In an additional experiment, Caco-2 cells were exposed to apigenin-quercetin mixtures at concentrations of 0.01 to 1.0  $\mu$ M (corresponding to concentrations used in Chapter 4) and cyclosporine A (10  $\mu$ M) for 6 h to determine the

potential nature of interactive effect of sorghum-cowpea mixtures in modulating ABC transporter proteins gene expression.

**Table 9:** The experimental design for the sorghum and cowpea phenolic extracts treatments.

	<b>Treatment group</b>	<b>Concentrations (µg/ml)</b>	<b>Functions</b>
<b>1</b>	<b>DMSO</b>		<b>Negative Control</b>
<b>2</b>	<b>Cyclosporin A</b>	<b>10 µM</b>	<b>Positive Control</b>
<b>3</b>	<b>White sorghum</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>4</b>	<b>White cowpea</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>5</b>	<b>White sorghum/white cowpea (1:1)</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>6</b>	<b>White sorghum/white cowpea (1:3)</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>7</b>	<b>White sorghum/white cowpea (3:1)</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>

**Table 10:** The experimental design for the apigenin and quercetin treatments.

	<b>Treatment group</b>	<b>Concentrations (µM)</b>	<b>Functions</b>
<b>1</b>	<b>DMSO</b>		<b>Negative Control</b>
<b>2</b>	<b>Cyclosporin A</b>	<b>10 µM</b>	<b>Positive Control</b>
<b>3</b>	<b>Apigenin</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>4</b>	<b>Quercetin</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>5</b>	<b>Apigenin/Quercetin (1:1)</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>6</b>	<b>Apigenin/Quercetin (1:3)</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>
<b>7</b>	<b>Apigenin/Quercetin (3:1)</b>	<b>0.005, 0.01, 0.05, 0.1, 1</b>	<b>Treatment</b>

#### *RNA Extraction and Real-Time PCR Analysis of mRNAs*

Total RNA was extracted using the Qiagen extraction kit (Qiagen Inc. Valencia, CA) based on the manufacturer's protocol. The NanoDrop<sup>®</sup> ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmington, DE) were used to evaluate the quality and quantity of isolated RNA.

### *mRNA Analysis*

A Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) was used to synthesize complementary DNA (cDNA) from the isolated RNA based on the manufacturer's protocol. Real Time PCR (qRT-PCR) was performed with the SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA).

### *Primer Sequences Used for mRNA Analysis*

The sequences of primers used were as follows:

BCRP (ABCG2):	F:	5'-TTTCAGCCGTGGAAGTCTTT-3'
BCRP (ABCG2):	R:	5'-TGAGTCCTGGGCAGAAGTTT-3'
MRP2 (ABCC2):	F:	5'-GACTATGGGCTGATATCCAGTGT-3'
MRP2 (ABCC2):	R:	5'-AGGCACTCCAGAAATGTGCT-3'
MDR1 (ABCB1):	F:	5'-CTCATCGTTTGTCTACAGTTCGT-3'
MDR1 (ABCB1):	R:	5'-GCTTTCTGTCTTGGGCTTGT-3'
MRP3 (ABCC3):	F:	5'-GGCACTGCTGATTGAAGACA-3'
MRP3 (ABCC3):	R:	5'-AATGGCTGCTTTCTCCTCCT-3'

Breast cancer resistance protein (BCRP/ABCG2), multi drug resistance protein

(MRPs/ABCCs), and p-glycoprotein (*P*-gp/MDR1/ABCB1) are apically located trans-membrane proteins that are responsible for apical transport of flavonoids metabolites across membranes (18, 19). Among the multidrug resistance proteins, MRP2 followed by MRP3 are the most abundantly expressed in human intestinal epithelial Caco-2 cell monolayers. MDR1 is the most expressed among the p-glycoprotein group in human intestinal epithelial Caco-2 cell monolayers (127).

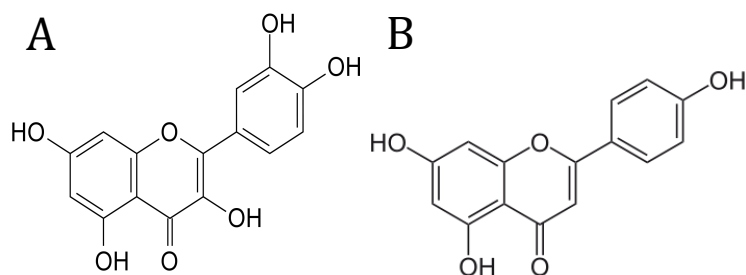
### *Protein Analysis*

Cells were seeded ( $5 \times 10^4$  cells/well) in 6-well plates. Seeded cells were grown and differentiated to confluent monolayers for 15-23 days before subjecting them to white sorghum-white cowpea treatments for 24 h. Prior the experiment, TEER values were measured to monitor the intensity and confluence of monolayers as previously described. In an additional experiment, Caco-2 cells were exposed to apigenin-quercetin mixtures (dominant flavonoids in white sorghum and white cowpea) at 0.01  $\mu$ M (the minimum effective concentration that showed synergistic effect in mRNA analysis results) to determine the potential nature of interactive effect of sorghum-cowpea mixtures in modulating ABC transporter proteins protein expression. Cells were harvested and cell lysates were obtained using the Pierce Ripa buffer (25mM Tris®HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) supplemented with Halt protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL.). Protein content was assessed using the Bradford reagent (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Proteins were separated on a 10 and 20% SDS-PAGE at 120 V

for 1 h. and transferred to PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (27). Membranes were blocked with 5% non- fat milk in 0.1% Tween-PBS (T-PBS) solution for 1 h. and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in T-PBS overnight at 4C with gentle shaking. After 3 washing steps with T-PBS for 5 minutes each, membranes were incubated with secondary antibodies (1:2000) in 5% non-fat milk dissolved in T-PBS for 1 h. Membranes were washed three times for 15 min and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

#### *Statistical Analysis*

Three replication of each treatment were performed. Analysis was using 2009 SAS (Version 9.3, SAS Inst. Inc., Cary, N.C., U.S.A.) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) was used to compare treatments means. Significant levels were defined using  $p < 0.05$ .



**Figure 37:** Skeletal structure of primary flavonol in white cowpea (quercetin) (A), and primary flavone in white sorghum (apigenin) (B).



## Results and Discussion

### *Effects of White Sorghum-White Cowpea Flavonoid Mixtures on P-glycoprotein (MDR1/ABCB1) Gene and Protein Expression*

#### Gene Expression

Caco-2 monolayers were used to investigate whether the white sorghum-white cowpea combinations had synergistic modulation against p-glycoprotein (*P*-gp) at concentration of 0.01 to 1.0 µg extract/mL. Sorghum-cowpea extract combinations demonstrated significantly ( $P<0.05$ ) higher down regulation in MDR1 gene expression than the additive effect of individual extracts relative to the control (untreated cells) in a dose dependent manner (Fig.2, 3). For example: the white sorghum-white cowpea mixture was more potent in modulating MDR1 gene expression (73%) compared to the additive effects of individual extracts (26%) at 0.05 µg/mL (Fig. 38).

Corresponding to the previous results in Chapter 4, sorghum-cowpea mixtures in different ratios (at similar concentrations), exhibited significantly ( $P<0.05$ ) different down regulation in MDR1 in mRNA level (Fig.2). For example: at 0.05 µg/mL concentration, mixture with (1:3) ratio (sorghum: cowpea) displayed stronger reduction (73%) in MDR1 gene expression than the mixture with (3:1) ratio (44%) (Fig. 38). Such dependency of synergistic inhibitory capacity on relative proportions suggests different mechanisms of action for sorghum and cowpea extracts. As previously mentioned,

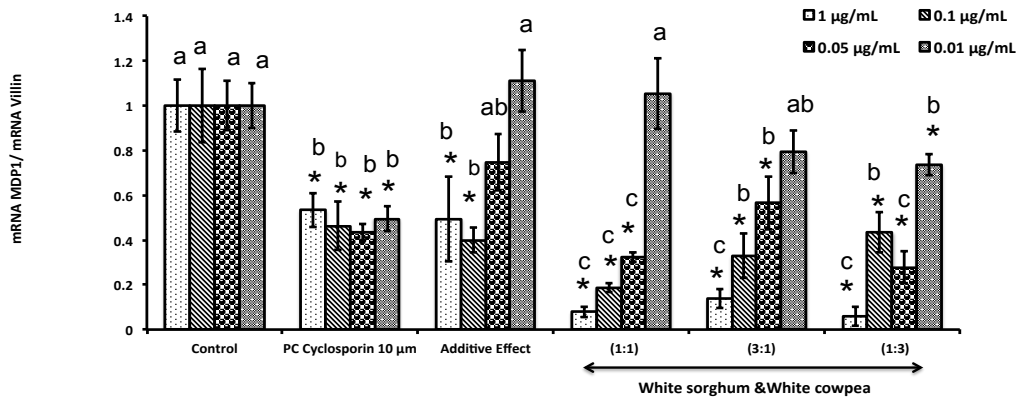
applying different ratios of sorghum and cowpea extracts resulted in treatments with different concentrations of putative active compounds. Thus, the dependency of synergistic inhibitory role on relative proportions could probably be due to non-similar operation of the active flavonoids in two commodities. Due to the complexity of sorghum-cowpea extracts, it is not possible to establish how specific compounds are interacting to suppress gene expression of transporter proteins. For this reason, we tested the dominant flavonoids compounds in each to gain insight on the additional interactive effects discussed in the later section.

The minimum effective dose that showed significant mitigation in MDR1 gene expression for additive effect of individual extracts was 0.1  $\mu\text{g/mL}$  (Fig. 38); whereas the sorghum-cowpea mixtures significantly ( $P<0.05$ ) inhibited MDR1 gene expression at 0.01  $\mu\text{g/mL}$  in the 1:3 mixture treatment (Fig. 39), which indicates that a more pronounced inhibition could be achieved at lower concentrations of different bioactive compounds combinations present in extracts versus the individuals compounds.

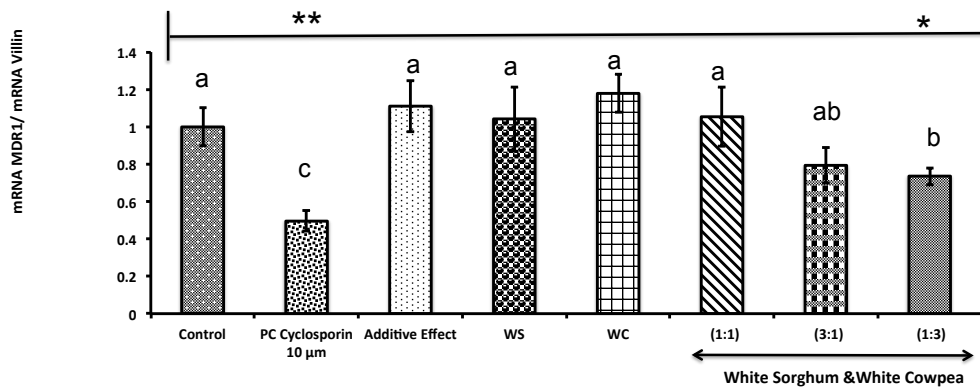
### Protein Expression

In order to test whether the inhibition of the mRNA expression of MDR1 by sorghum-cowpea mixtures was correlated to the modulation of protein levels, we measured MDR1 protein expression by western blot analysis at 0.01  $\mu\text{g}$  extracts/mL (equivalent to average of 80.19 ng GAE/mL in white sorghum/white cowpea extracts), which was the minimum effective dose that showed significant inhibitory effect in MDR1 gene expression results (Fig. 39). The western blot analysis revealed stronger

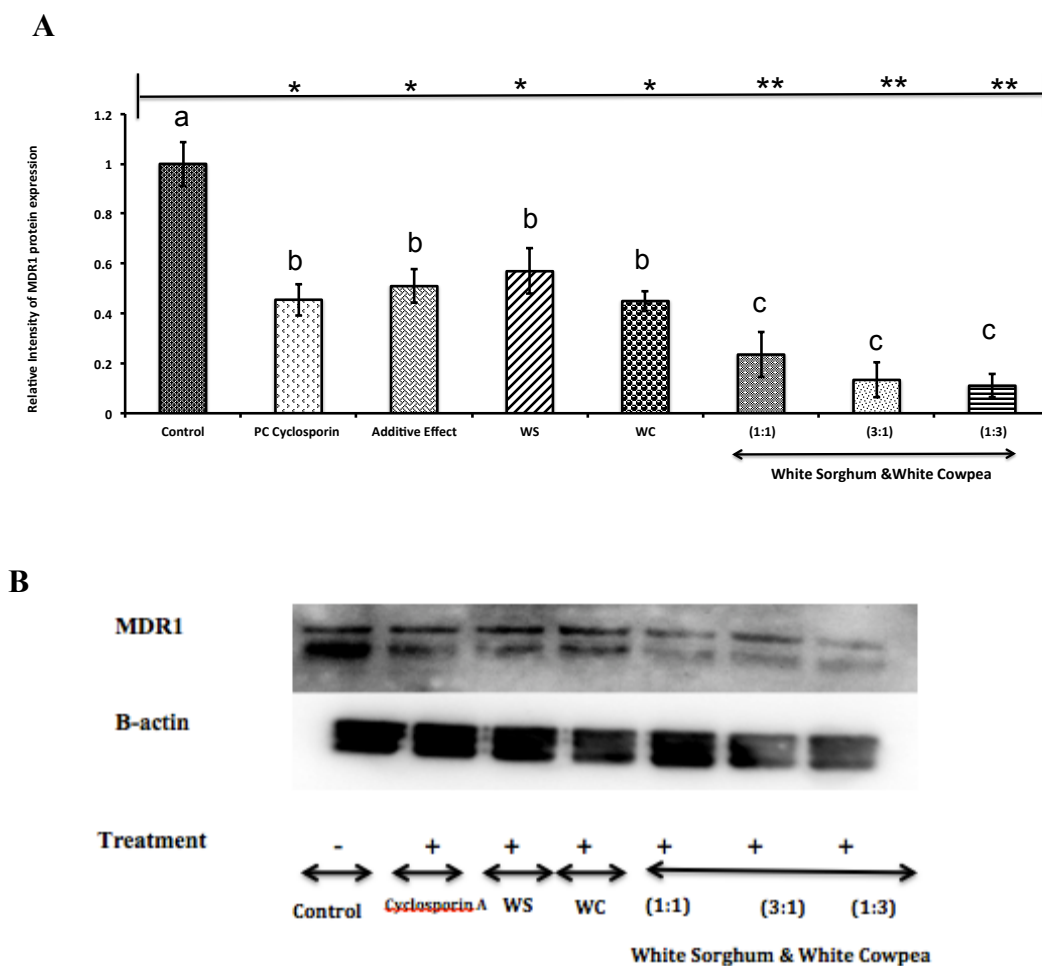
reduction in MDR1 protein expression (90%) than the additive effect (40%) at the same concentration (Fig. 40). The white sorghum-white cowpea combinations (1:1, 3:1, 1:3) also displayed significantly ( $P<0.05$ ) higher inhibitory rates against MDR1 protein expression versus the positive control (cyclosporine A). These results suggest the capacity of flavonoid mixtures to modulate MDR1 protein expression at lower concentrations than tested in this study. Comparable to the gene expression results, the mixtures ratio significantly affected magnitude of synergistic inhibitory effect. For example: the combination in 1:3 ratio (90%) was more effective in reducing MDR1 protein expression compared to the combination in 1:1 ratio (77%) (Fig. 40). Interestingly, the 1:3 ratio also showed the most effective inhibitory effect on MDR1 gene expression results.



**Figure 38:** Effect of extracts of sorghum and cowpea mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/*P*-gp) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01-1.0 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 39:** Effect of extracts of sorghum and cowpea mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/ *P*-gp) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 40:** Effect of extracts of sorghum and cowpea mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/ *P*-gp) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with extracts (0.01  $\mu\text{g/mL}$ ) of sorghum and cowpea combinations; and analyzed by western blot as ratio to  $\beta$ -actin protein (**A**). Western blot analysis of MDR1 (**B**). Values are means  $\pm$  SD ( $n = 3$ ). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

P-glycoprotein (*P*-gp), encoded by the ABCB1 gene, is the first human ABC transporter that was cloned and characterized through its ability to confer a multi-drug resistant phenotype to cancer cells. *P*-gp is mainly responsible for outward transportation

(i.e., efflux) of hydrophobic, cationic or neutral compounds (61). Among the *P*-gp group, multi-drug resistant protein1 (MDR1) was included in this study due to its highest expression level in human intestinal epithelial Caco-2 cell monolayers (127). Various flavonoids including quercetin, hesperitin, naringenin, kaempferol, chrysin and apigenin (10  $\mu$ M) are known to inhibit *P*-gp protein with different potencies and efficacies by several mechanisms such as directly interacting with the ATP binding sites (49, 81, 112). However, in most cases their corresponding glycosides did not show the same efficiency against *P*-gp protein (49, 81, 112).

Conflicting results regarding the flavonoids- *P*-gp interactions have been reported in literature, which could be due to the structure-activity relationship between flavonoids and *P*-gp that influence the binding properties of the model used for each study (3, 17, 49). Shape parameter and hydrophobicity are two major physico-chemical parameters that are responsible for the affinity of flavonoid derivatives towards *P*-gp sites (17, 49). For example: the flavonol group (quercetin and kaempferol) depending on their concentrations has shown a biphasic effect on vincristine (a chemotherapy medication used to treat a number of cancer cells) efflux, causing inhibition or enhancement (3).

Until now, we are not aware of any studies that have determined the synergistic effect of phytochemical (flavonoid) combinations on *P*-gp protein modulation. This is the first study reporting the enhanced action in modulation of *P*-gp genes and proteins by natural complementary flavonoid mixtures with practical relevance to diet. The evidence suggests a strong complementary nature of flavonoids substituted and un-substituted at position 3 in C-ring against *P*-gp expression.

The main flavonoid compounds identified in sorghum and cowpea; apigenin and quercetin, have been separately shown to inhibit *P*-gp protein with different potencies and efficacies in human cell models through multiple mechanisms (3, 61, 113, 132). Therefore, our study suggests that the co-administration of structurally complementary natural flavonoids present in sorghum and cowpea, targeting different signaling mechanisms has the potential to increase competitive inhibitory action on *P*-gp. This indicates that lower doses of each commodity, which is easily achievable through habitual diet, can be consumed to provide significant benefits.

*Effects of White Sorghum-White Cowpea Flavonoid Mixtures on Multi Drug Resistant Protein (MRP2/ABCC2, MRP3/ABCC3) Gene and Protein Expression*

MRPs is an efflux transporter, which belongs to the ABCC (also called MRP) subfamily of ABC transporters (61). The main function of MRPs, which is located to apical membrane of polarized cells, is to actively transport endogenous (e.g. bilirubin and its polar conjugates) and xenobiotic (e.g. drugs) substances out of the cells (61). Among the multidrug resistance proteins, MRP2 followed by MRP3 are the most abundantly expressed in human intestinal epithelial Caco-2 cell monolayers (127).

Corresponding to the *P*-gp results, sorghum-cowpea extract combinations displayed significantly ( $P < 0.05$ ) higher reduction in both MRP2 and MRP3 expression compared to the additive effect of individual extracts at mRNA level relative to the control (untreated cells) (Fig. 41, 44). For example: higher down regulation 87% in MRP2 gene expression was exhibited by the sorghum-cowpea extract combination compared to the

additive effects of individual extracts (14%) at 0.05 µg/mL (Fig. 41).

As observed with *P*-gp, the ratio of sorghum: cowpea had significant effect on magnitude of synergy in modulation of MRP2 and MRP3 in mRNA and protein expression (Fig. 41-46). For instance: at 0.05 µg/mL concentration, mixture with (3:1) ratio showed stronger reduction (87%) in MRP2 gene expression than the mixture with (1:1) ratio (67%) (Fig. 41). The treatment in 1:1 ratio showed stronger down regulation (82%) in MRP3 protein expression than the treatment in 1:3 (57%) (Fig. 46).

The mixture in 3:1 ratio revealed the most potent inhibition in both MRP2 and MRP3 gene expression; whereas for MDR1 (*P*-gp) in previous Section, the 1:1 ratio showed the highest inhibitory effect. These disagreements in flavonoids efficiency between *P*-gp and MRPs proteins could be due to the different structure-activity relationships (binding site conformations) in each protein that affect the flavonoid-transporter proteins interactions.

The minimum effective dose that showed significant mitigation in MRP2 and MRP3 gene expression for additive effect of individual extracts was 0.1 µg/mL (Fig. 41, 44); whereas the sorghum-cowpea mixtures significantly ( $P < 0.05$ ) inhibited MRP2 and MRP3 gene expression at 0.01 µg/mL (Fig. 42, 45). The magnitude of synergy (10 times) was consistent with the *P*-gp (MDR1) data (Fig. 38).

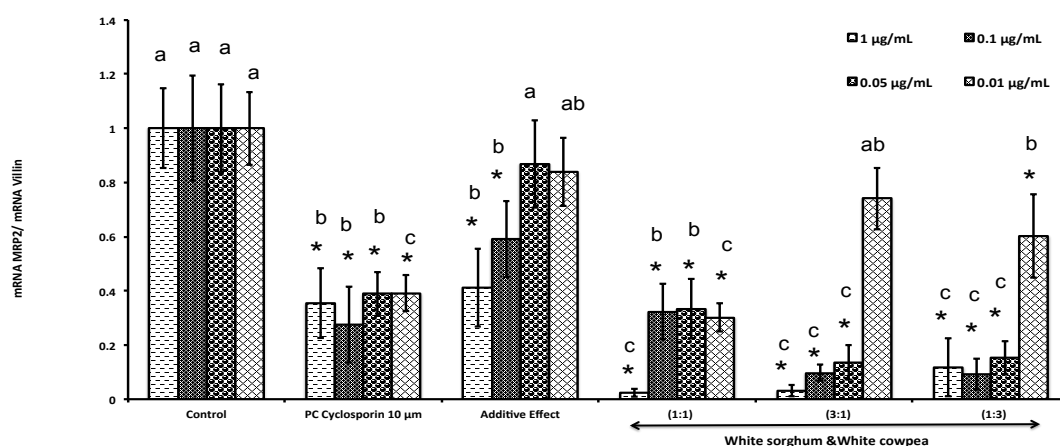
Western blot analysis demonstrated that at lowest effective dose (0.01 µg/mL) that showed inhibitory effect in gene expression results, sorghum-cowpea mixtures were more potent in modulation of both MRP2 and MRP3 protein expression compared to additive effects (Fig. 43, 46), which confirmed correlation between gene and protein expression results. The correlation was also in agreement with *P*-gp protein data. For



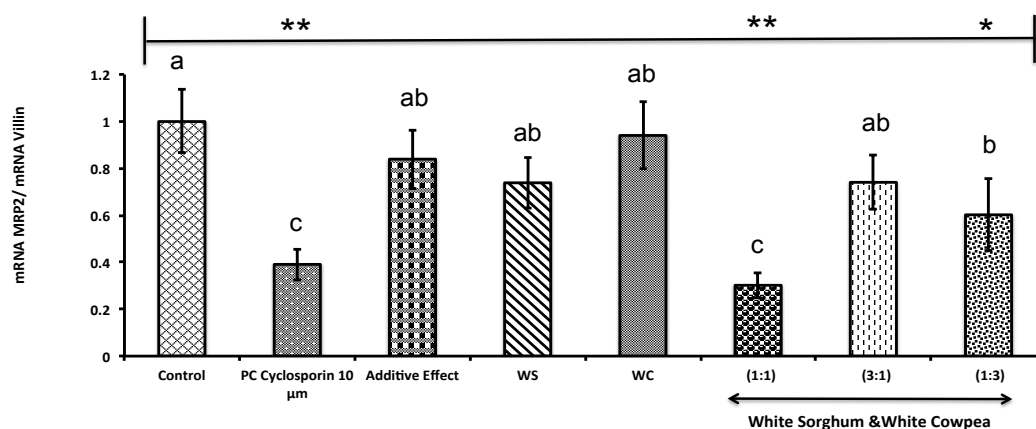
example, the mixture in 1:3 ratio revealed stronger suppression (91%) in MRP3 protein expression compared to the additive effect of individual extracts (12%) (Fig. 46).

Among 29 structurally different tested flavonoids, myricetin and robinetin were the two best MRP2 inhibitors in MDCKII cells, indicating that a flavonol B-ring pyrogallol group appeared to be an essential structure for inhibition of MRP2 protein (134). The only research that determine the effect of phytochemicals combinations on MRPs proteins (but at higher concentrations compared to our study), showed that the co-incubation of quercetin, chrysin, genistein and resveratrol, all in equal concentrations and ratios (50  $\mu$ M) increased ochratoxin A (a food-borne mycotoxin) cellular accumulation and transportation to the basolateral (blood) side in Caco-2 cells by almost 50% and 80% respectively (3). It was hypothesized that polyphenols may exert this effect through competitive inhibition of MRP2 (3).

In our study, due to the complexity of sorghum and cowpea matrixes, multiple interactions might take place simultaneously. MRP2 has at least two binding sites. Positive competitive or complementarily cooperation between flavonoids as well as between flavonoids and other minor bioactive compounds might have resulted in more effective modulation in sorghum-cowpea mixtures at such low concentrations. Quercetin derivatives (dominant flavonoid in white cowpea  $7.3 \pm 2.6$   $\mu$ g/mL) and apigenin (prevalent flavonoid in white sorghum  $5.4 \pm 0.12$   $\mu$ g/mL), due to the lack of the pyrogallol group, are not known as the most effective modulators of MRP2 proteins, but have been separately shown to inhibit MRP2 protein with different potencies and efficacies through multiple mechanisms (3).

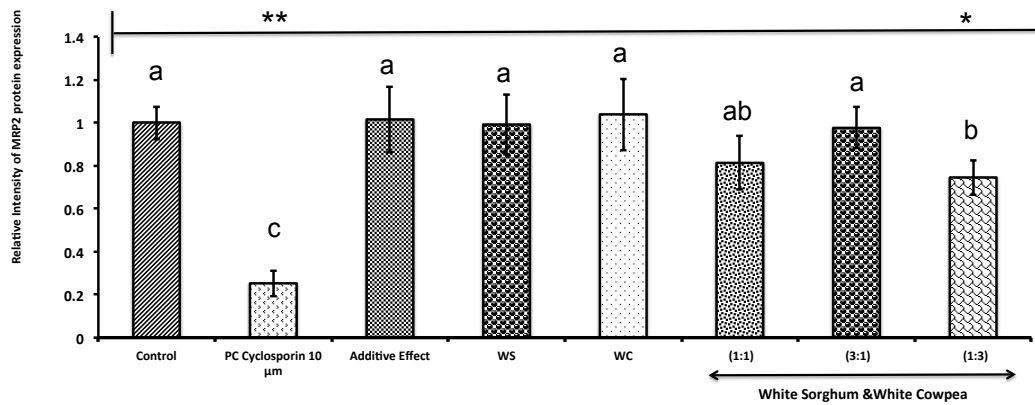


**Figure 41:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 2 (MRP2) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01-1.0 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

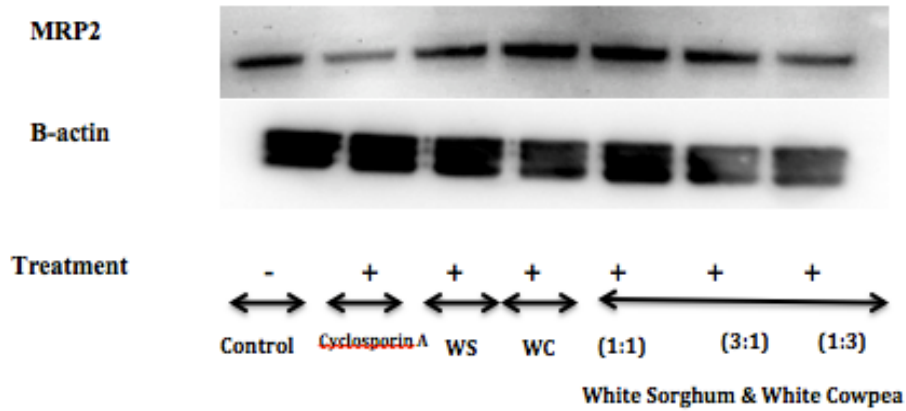


**Figure 42:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 2 (MRP2) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

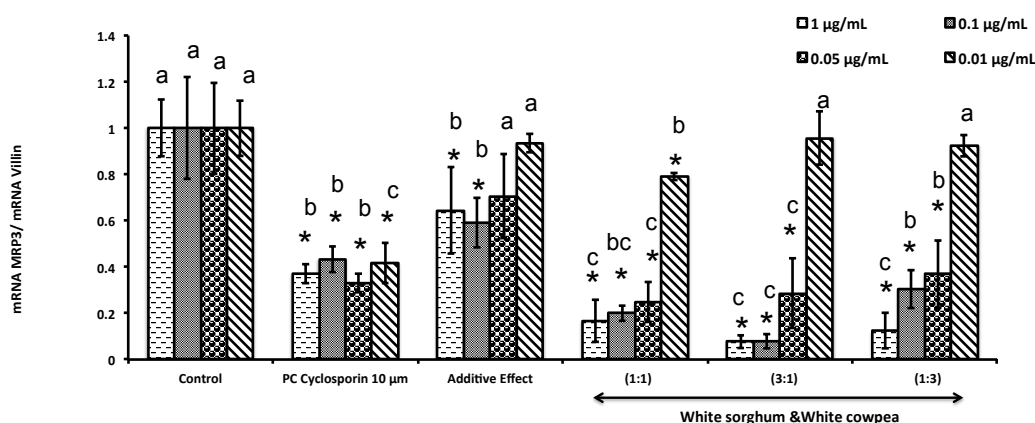
**A**



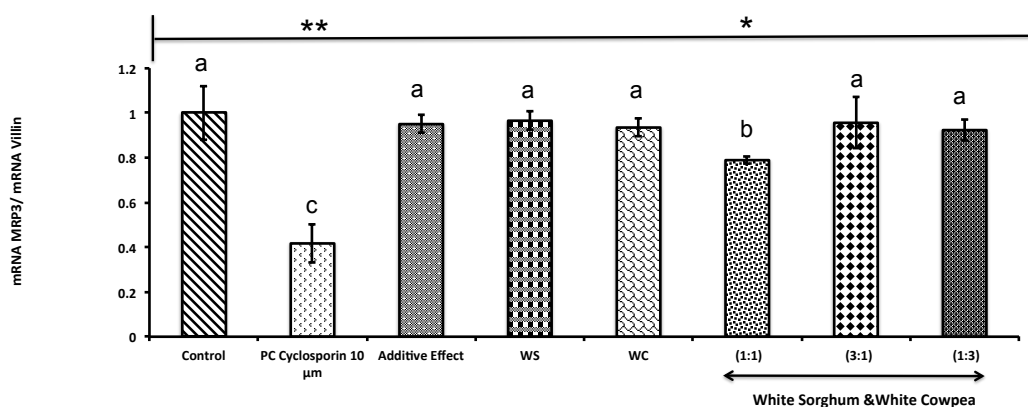
**B**



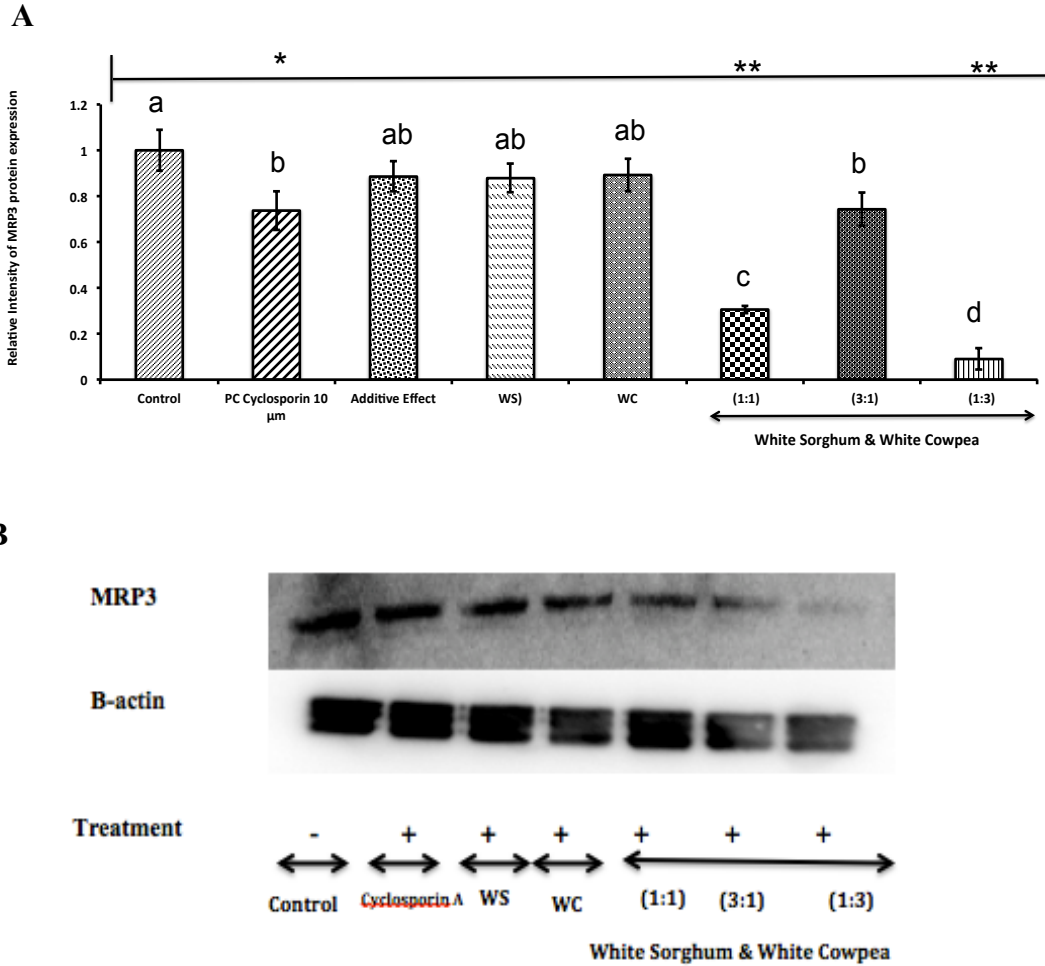
**Figure 43:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 2 (MRP2) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with extracts (0.01 µg/mL) of sorghum and cowpea combinations; and analyzed by western blot as ratio to  $\beta$ -actin protein (A). Western blot analysis of MRP2 (B). Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 44:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 3 (MRP3) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01-1.0 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 45:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 3 (MRP3) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 46:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 3 (MRP3) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with extracts (0.01  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot as ratio to  $\beta$ -actin protein (**A**). Western blot analysis of MRP3 (**B**). Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

*Effects of White Sorghum-White Cowpea Flavonoid Mixtures on Breast Cancer Resistance Protein (BCRP/ ABCG2) Gene and Protein Expression*

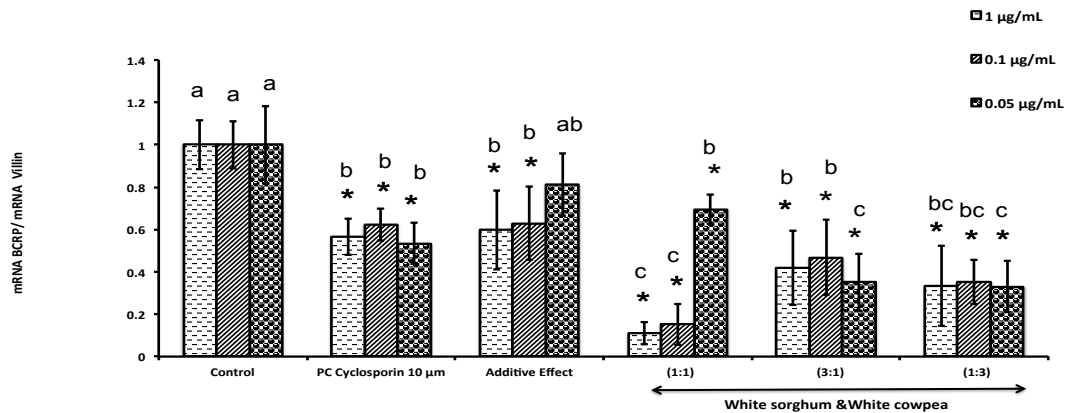
Breast cancer resistance protein (BCRP), which is encoded by the ABCG2 gene, has an important role in limiting oral bioavailability of drugs and drug transport across the blood– brain barrier, blood–testis barrier and the maternal–fetal barrier of selected substrates (61). BCRP is broadly distributed in humans including kidney, liver and intestine (61). The substrates of BCRP include various compounds from endogenous to exogenous, which are generally organic anion or neutral substances (61).

Sorghum-cowpea extract combinations generally showed significantly ( $P<0.05$ ) stronger inhibitory role against BCRP expression than the additive effect of individual extracts at mRNA level (Fig. 47), which was consistent with MRPs and *P*-gp results (Fig. 38, 41, 44). Once more, the sorghum-cowpea mixture ratios significantly ( $P<0.05$ ) affected potency in reducing BCRP mRNA and protein expression (Fig. 47, 48). For example: at 0.05  $\mu\text{g/mL}$ , mixture with 1:3 ratio revealed more effective inhibitory role (67%) against BCRP gene expression than the mixture with 1:1 ratio (31%) (Fig. 47). The combination with 3:1 ratio (61%) was more effective at reducing BCRP protein expression compared to the combination with 1:3 ratio (42%) (Fig. 49).

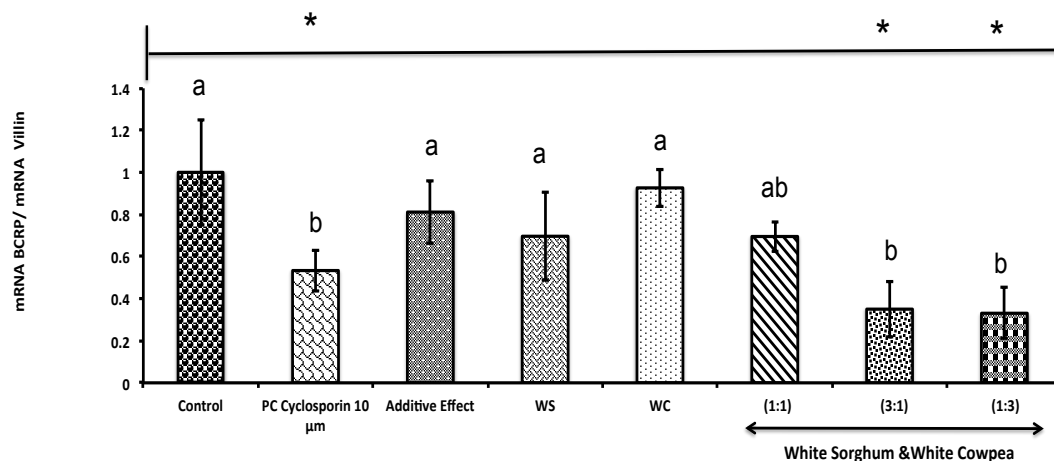
The minimum effective dose that showed significant mitigation in BCRP gene expression for additive effect of individual extracts was 0.1  $\mu\text{g/mL}$  (Fig. 47); whereas the sorghum-cowpea mixtures significantly ( $P<0.05$ ) inhibited BCRP gene expression at 0.05  $\mu\text{g/mL}$  in the mixture treatment (Fig. 48). The magnitude of synergy for BCRP was smaller than the magnitude of synergy for *P*-gp and MRPs (2 times versus 10 times), which is

probably again due to the differences in their binding site conformations as well as the essential structures for their inhibition that influence the flavonoid-transporter protein interactions.

Corresponding to MRPs and *P*-gp data, good agreement was observed between BCRP gene and protein results. The western blot analysis demonstrated stronger inhibition by sorghum-cowpea mixtures against BCRP protein expression than the additive effect at lowest effective dose (0.05 µg/mL), that showed significant inhibitory effect in gene expression results, (Fig. 49). For example, the mixture in 3:1 ratio revealed stronger suppression (61%) of BCRP protein expression versus the additive effect of individuals (34%) (Fig. 49).

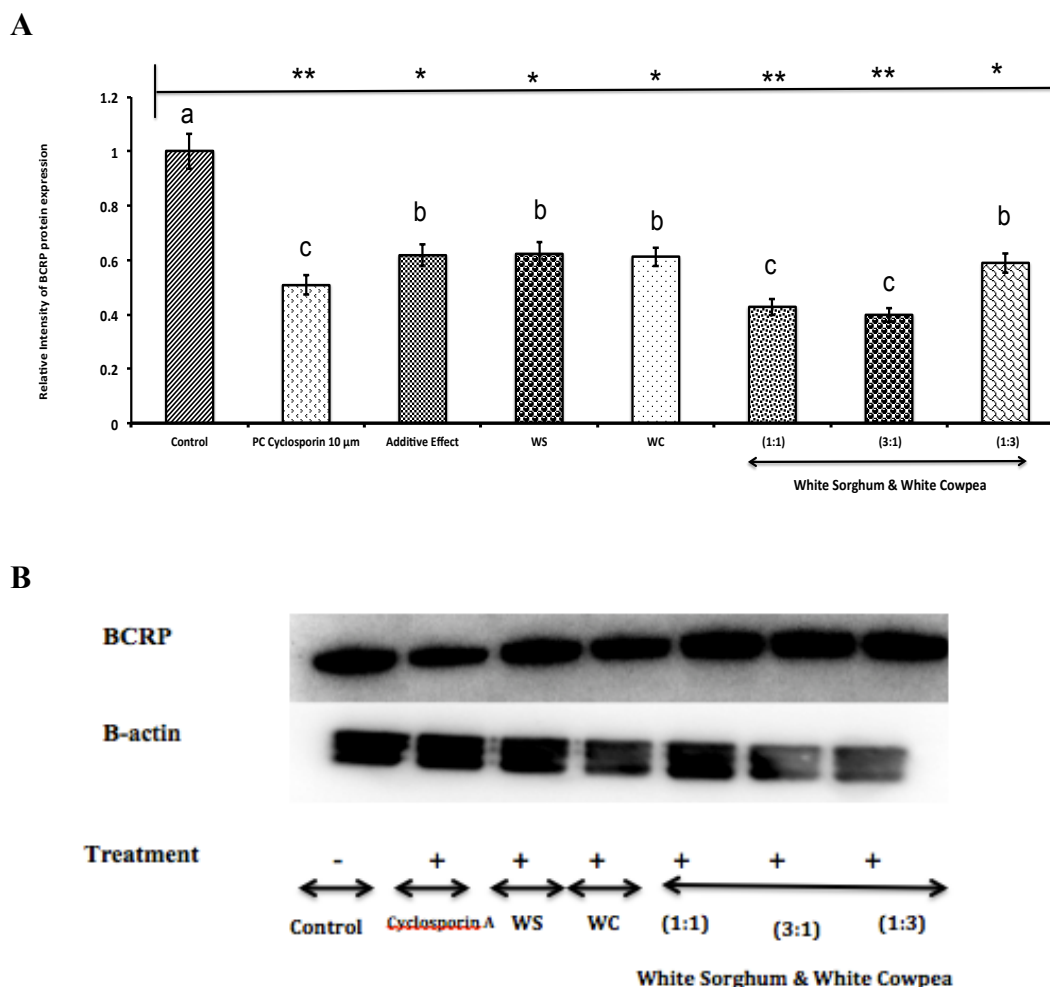


**Figure 47:** Effect of extracts of sorghum and cowpea mixtures on breast cancer resistance protein (BCRP) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.01-1.0 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 48:** Effect of extracts of sorghum and cowpea mixtures on breast cancer resistance protein (BCRP) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts (0.05 µg/mL) of sorghum and cowpea combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).





**Figure 49:** Effect of extracts of sorghum and cowpea mixtures on breast cancer resistance protein (BCRP) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with extracts (0.05 µg/mL) of sorghum and cowpea combinations; and analyzed by western blot as ratio to  $\beta$ -actin protein (**A**). Western blot analysis of BCRP (**B**). Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

The magnitude of synergy in modulating the transporter proteins gene and protein expression was dependent on the relative proportions of each extract in all transporter proteins tested (BCRP, MRP2, MRP3 and MDR1). The sorghum-cowpea combination with 1:1 ratio was the most potent treatment in reducing MDR1 gene expression at 0.05  $\mu\text{g/mL}$ ; whereas for MRP2 the mixture with 3:1 ratio, and for BCRP the mixture with 1:3 ratio, revealed the most effective inhibitory role at the same concentration. Since different flavonoid's structure affect transporter proteins differently, such complex interactions of different extract ratios on the transporter protein and gene expression are expected. Changing the ratios of sorghum-cowpea mixtures alters concentrations of relative compounds, thus altering interactive effects on each protein differently. Due to the complexity of sorghum-cowpea extracts and their multifaceted mutual interactions with transporter proteins, it is not possible to designate one ratio as the most effective in suppressing expression of transporter proteins. The most important finding, however is that the mixtures were consistently more potent than the additive effect regardless of ratio or concentration, with synergistic effect of between 2-10 fold observed.

Among all the ABC transporters, the inhibitory role of flavonoids (apigenin, genistein, kaempferol, naringenin, hesperitin) (50  $\mu\text{M}$ ) against BCRP has been investigated (24, 57, 101, 128, 146). A few studies have reported synergistic inhibition of BCRP by flavonoid mixtures (17, 18, 19, 148), but these studies are mostly limited to purified polyphenol mixtures at higher concentrations compared to our study. For example: Zhang *et al.*, 2004 found synergistic inhibitory effect against BCRP in mixtures of apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin,

naringenin, and silymarin for increasing mitoxantrone accumulation when given as 2-, 3-, 5-, or 8-flavonoid combinations in equal molar concentration (10-50  $\mu$ M). The combined effects of multiple flavonoids reduced the BCRP expression in a wide range (10-90%), based on the type of flavonoids and resulted in increasing mitoxantrone accumulation (148). Another study demonstrated that multiple flavonoid combinations including kaempferide, biochanin A, 5,7- methylflavone and 8-methylflavone in equal molar concentration (2.5-5  $\mu$ M) of each individual constituent synergistically inhibited BCRP expression (10 fold) versus the individual flavonoids which lead to increasing mitoxantrone accumulation in breast cancer cells (4).

In our study, sorghum-cowpea mixtures synergistically modulated the expression of ABC transporters (BCRP, MRP2, MRP3, MDR1) at concentrations of 0.01 to 1.0  $\mu$ g extracts/mL at both gene and protein levels, which demonstrates that the sorghum-cowpea combinations are effective in both transcriptional and translational steps at concentrations with practical relevance to diet. Strong and consistent synergy in inhibitory role of flavonoid mixtures from white sorghum and white cowpea against efflux transporters in both genomic and proteomic tests demonstrate the complementary potential of natural products with distinctly different flavonoids profiles in enhancing potential bioavailability (and by extension bioactivity) of flavonoids and their metabolites.

Apigenin was shown to modulate ABC transporters in human cell models. For example: apigenin (20  $\mu$ M) inhibited both *P*-gp and BCRP by competing with ATP for binding sites leading to energy depletion to fuel the transport of ABC transporter

substrates, which resulted in increasing cellular uptake of doxorubicin (a drug used for cancer) in multidrug resistant cells (113). In the same study, the combination of apigenin (20  $\mu$ M) with docetaxel (1  $\mu$ M) (chemotherapy medication) showed synergistic inhibition (65%) in *P*-gp expression compared to the individuals (25%) in HEK293 ABCB5 cells (113). Quercetin also inhibited *P*-gp in a dose dependent manner (10-100  $\mu$ M) by binding to one of the two substrate (drug) binding sites; this resulted increasing in anti-cancer drug adriamycin accumulation in HCT-15 colon carcinoma cells (82).

No studies have determined the interactions of co-administration of flavone and flavonol groups on efflux transporters. Since different flavonoids structures affect transporter proteins differently, apigenin and quercetin are expected to operate differently in suppressing the expression of transporter proteins (101, 146). Hence, the combination of these compounds could be responsible for the enhanced health benefits of sorghum-cowpea mixtures. Apigenin and quercetin mixtures were tested to further determine the potential contribution of flavones and flavonols in the synergistic effect of sorghum-cowpea mixtures against ABC transporters.

#### *Effects of Apigenin-Quercetin Mixtures on ABC Transporters (MDR1, MRP2, MRP3 and BCRP) Gene and Protein Expression*

The apigenin-quercetin combinations synergistically modulated the ABC transporter's (MDR1, MRP2, MRP3 and BCRP) gene expression at concentrations of 0.01 to 1.0  $\mu$ M (Fig. 50, 53, 56, 59), which was in agreement with the synergistic

inhibitory effect of sorghum-cowpea mixtures against the same transporter proteins (Fig. 38, 41, 44, 47).

Corresponding to the sorghum-cowpea mixtures data, apigenin-quercetin combinations with different ratios (1:1, 1:3, 3:1) (at similar concentrations), displayed significantly ( $P<0.05$ ) different inhibitory effect in all ABC transporter proteins tested at both mRNA and protein levels (Fig. 50-61). For example: the combination in 1:1 ratio was significantly ( $P<0.05$ ) more effective (75%) in inhibiting MDR1 gene expression compared to the combination in 1:3 ratio (49%) at 1.0  $\mu\text{M}$  (Fig. 50). The combination in 1:3 ratio was more effective (57%) in reducing BCRP protein expression compared to the combination in 1:1 ratio (12%) (Fig. 61). These results confirm that the flavone/flavonol mixtures can synergistically down-regulate ABC transporters.

The magnitude of synergy was similar to the sorghum-cowpea results for *P*-gp, MRPs (10 fold) as well as BCRP (2 fold). The minimum effective dose that revealed significant inhibition of *P*-gp, MRPs and BCRP gene expression for additive effect of individual extracts was 0.1  $\mu\text{M}$  (Fig. 50, 53, 56, 59); whereas the apigenin-quercetin mixtures significantly ( $P<0.05$ ) down regulated *P*-gp and MRPs gene expression at 0.01  $\mu\text{M}$  and BCRP gene expression at 0.05  $\mu\text{M}$  (Fig. 51, 54, 57, 60).

Western blot analysis revealed that reduction of ABC transporters mRNA expression by apigenin-quercetin mixtures was correlated to the mitigation of protein levels. Apigenin-quercetin mixtures exhibited synergistic reduction in *P*-gp, MRPs and BCRP protein expression at the minimum effective doses that revealed synergistic effect in *P*-gp, MRPs and BCRP gene expression results (0.01  $\mu\text{M}$ , 0.01  $\mu\text{M}$ , and 0.05  $\mu\text{M}$

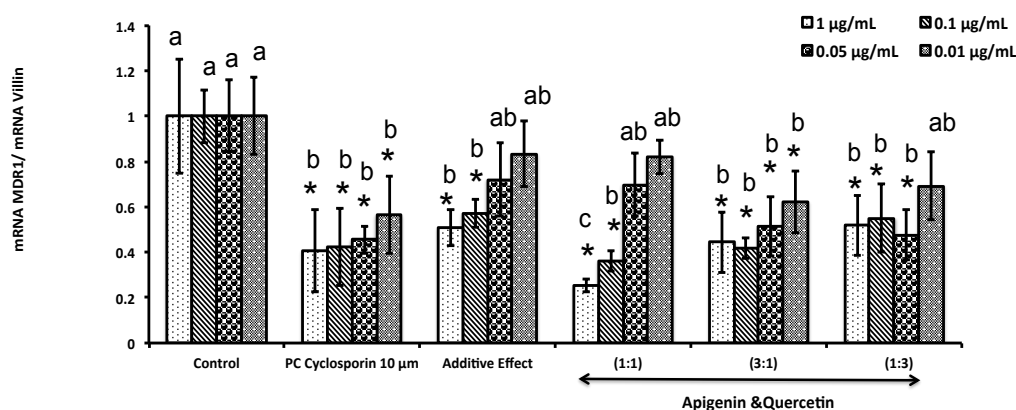
respectively) (Fig. 52, 55, 58, 61). For example: the apigenin-quercetin combination in 1:3 ratio showed stronger modulation in MDR1 protein expression (45%) than the additive effect (10%) (Fig. 52).

The most important finding, however is that comparable to the sorghum-cowpea combinations, the apigenin-quercetin mixtures were consistently more effective in ABC transports modulation versus the additive effect regardless of ratio or concentration, with the same magnitude of synergy as the sorghum-cowpea mixtures (2-10 fold).

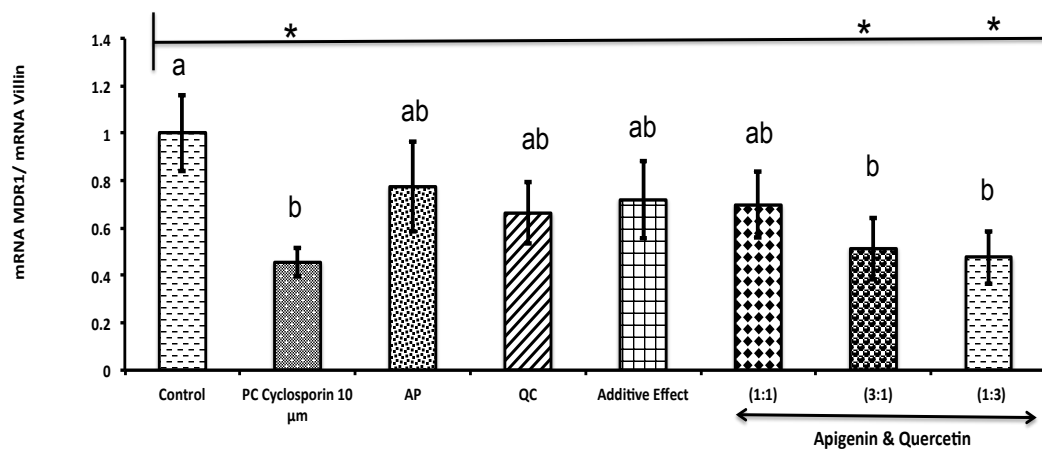
Although consistent synergy in down regulating ABC transporters gene and protein expression was observed in both sorghum-cowpea and apigenin-quercetin results ( $12.8 \pm 2.8$  mg flavonoids/g extract), the minimum effective concentrations that showed synergistic effect in sorghum-cowpea extracts (0.12-12 ng flavonoid/g extract) mixtures was much lower than apigenin-quercetin mixtures (2.85-285 ng flavonoids/g extract). Previous studies have demonstrated that using isolated/purified compounds may not be a good predictive model for establishing magnitude the bioactivity of flavonoids in foods (*143*). The potential activity of a given compound in a complex natural combination such as most foods might be higher than can predicted by applying purified compounds. A possible explanation is that in a natural mixture, sorghum-cowpea in this case, small quantities of diverse bioactive compounds may favorably interact to enhance ABC transporters modulation in complex ways.

Several studies have reported the inhibitory roles of flavonoids against ABC transporters in wide range of concentrations (10-250  $\mu M$ ) (*3, 20, 57, 82, 148*). In contrast, few studies have reported synergistic inhibitory role of flavonoid mixtures

against ABC transporters (4, 18, 19, 147). This is the first study reporting the enhanced action in modulation of efflux transporters genes and proteins by natural cereal-legume flavonoid mixtures. These effects were apparent at low concentrations relevant to diet. Overall, the findings in this study suggest interactive effect in transporters modulation revealed in sorghum-cowpea flavonoid mixtures, could be a mechanism for sorghum-cowpea enhanced action against inflammation discussed in Chapter 4.



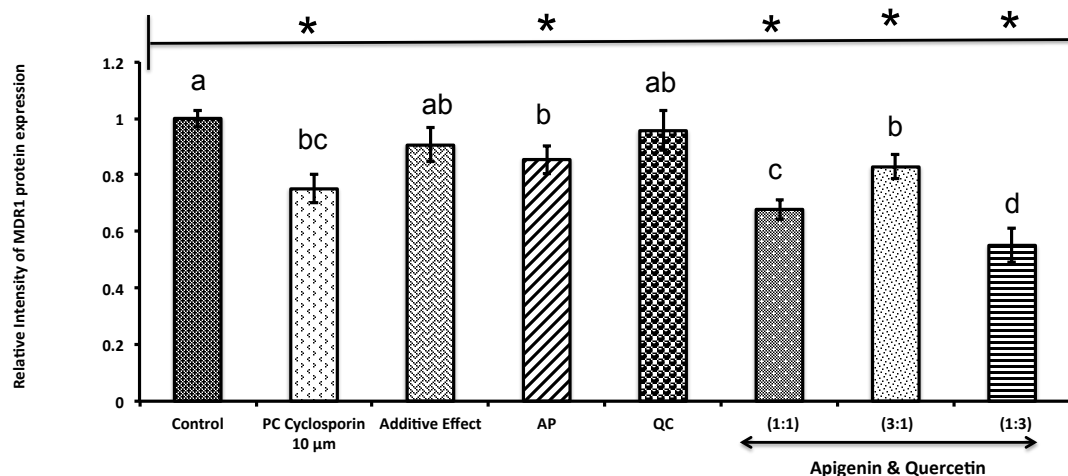
**Figure 50:** Effect of apigenin and quercetin mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/P-gp) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin combinations at 0.01-1.0 µM; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means ± SE (n = 3). (\*) indicate significant difference at p < 0.05 compared to control (non-treated cells). Different letters indicate significant difference at p < 0.05 compared to positive control (cyclosporine A-treated cells).



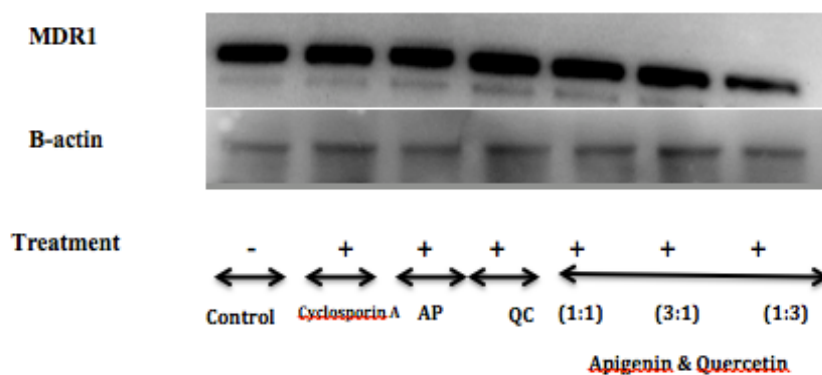
**Figure 51:** Effect of apigenin and quercetin mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/ *P*-gp) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin (0.01  $\mu$ M) combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



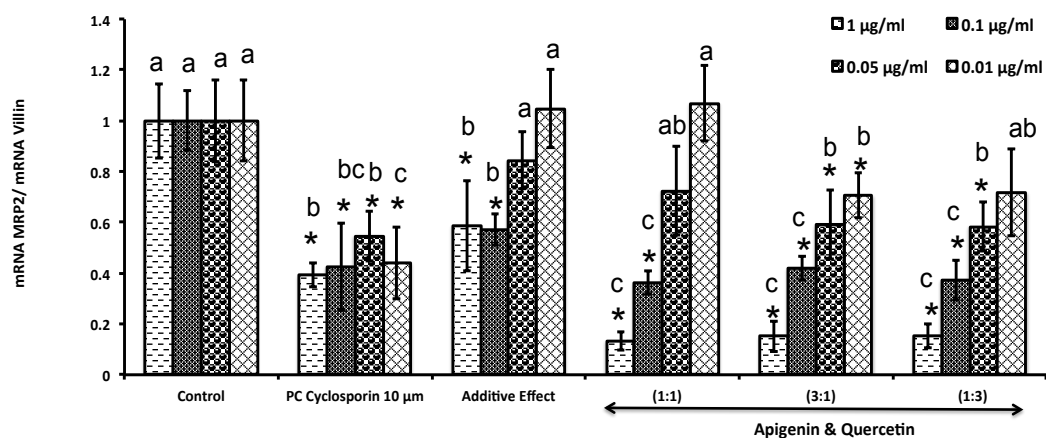
**A**



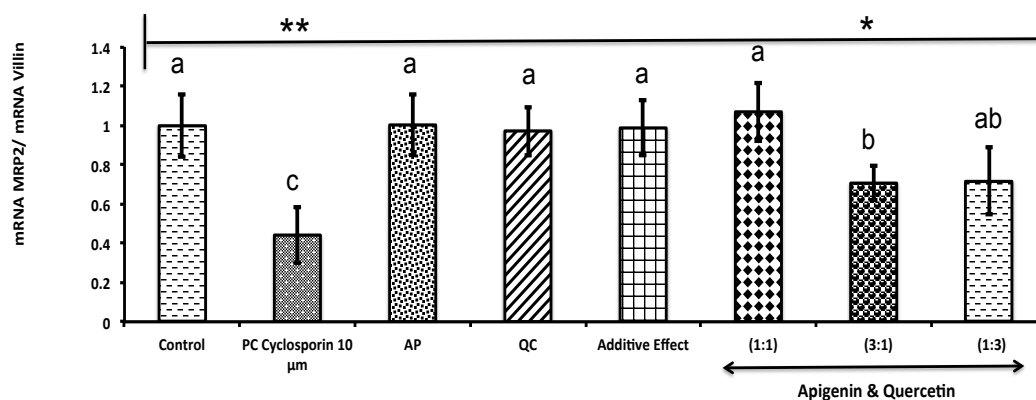
**B**



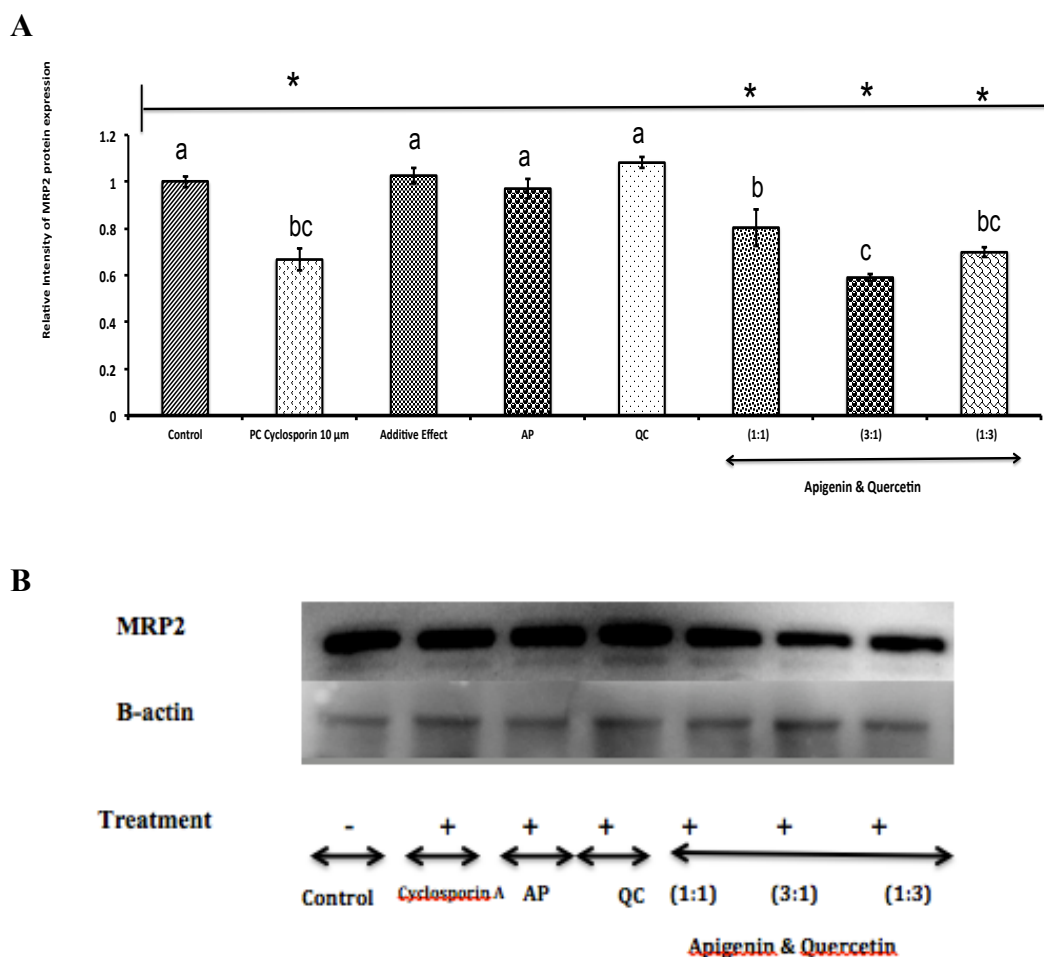
**Figure 52:** Effect of apigenin and quercetin mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/P-gp) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with apigenin and quercetin (0.01 µM) combinations and analyzed by western blot as ratio to β-actin protein (**A**). Western blot analysis of MDR1 (**B**). Values are means ± SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



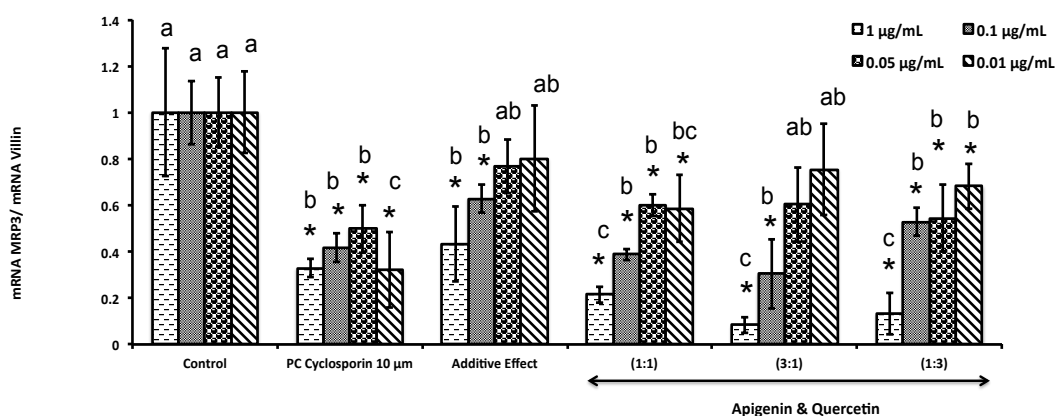
**Figure 53:** Effect of apigenin and quercetin mixtures on multidrug resistant associated protein 2 (MRP2) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin combinations at 0.01-1 µM; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



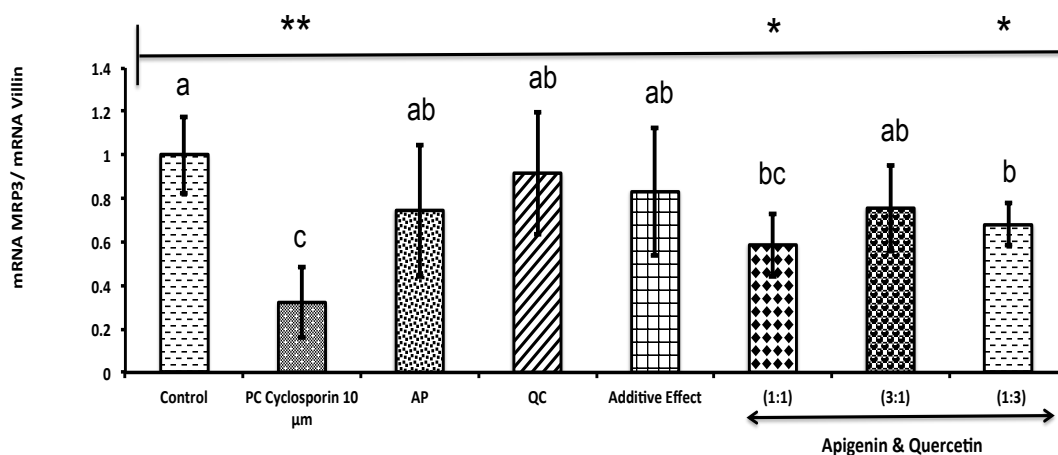
**Figure 54:** Effect of apigenin and quercetin mixtures on multidrug resistant associated protein 2 (MRP2) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin (0.01 µM) combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters within each assay indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).



**Figure 55:** Effect of apigenin and quercetin mixtures on multidrug resistant associated protein 2 (MRP2) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with apigenin and quercetin (0.01 µM) combinations; and analyzed by western blot as ratio to β-actin protein (A). Western blot analysis of MDR1 (B). Values are means ± SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

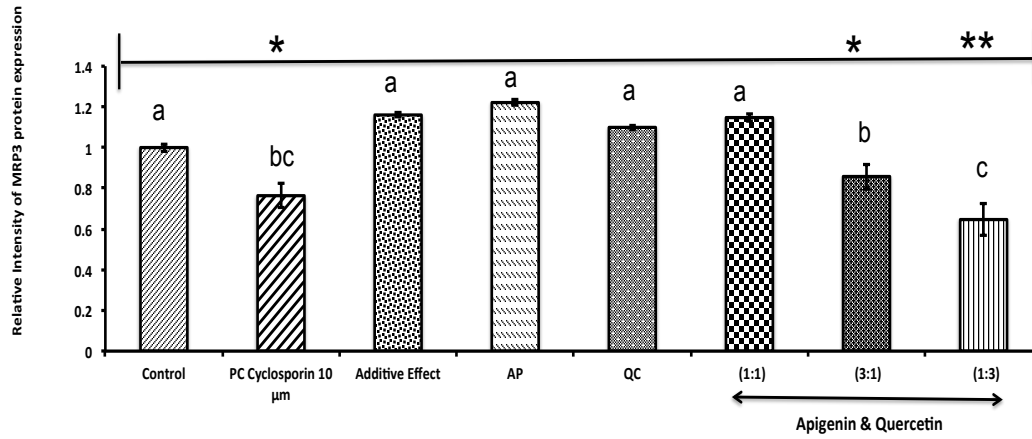


**Figure 56:** Effect of apigenin and quercetin mixtures on multidrug resistant associated protein 3 (MRP3) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin combinations at 0.01-1µM; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

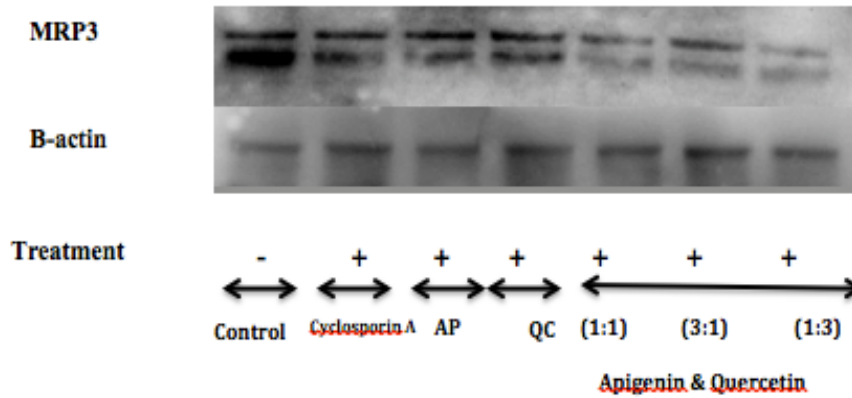


**Figure 57:** Effect of apigenin and quercetin mixtures on multidrug resistant associated protein 3 (MRP3) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin (0.01 µM) combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

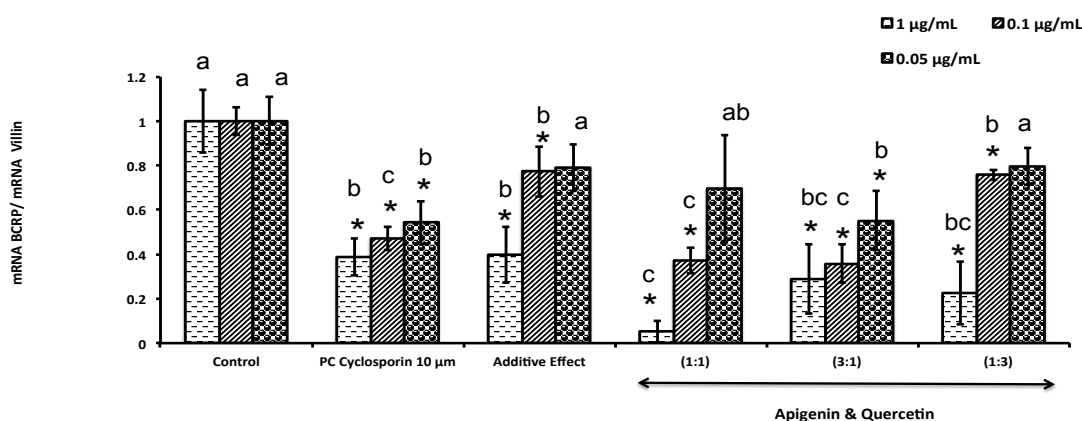
**A**



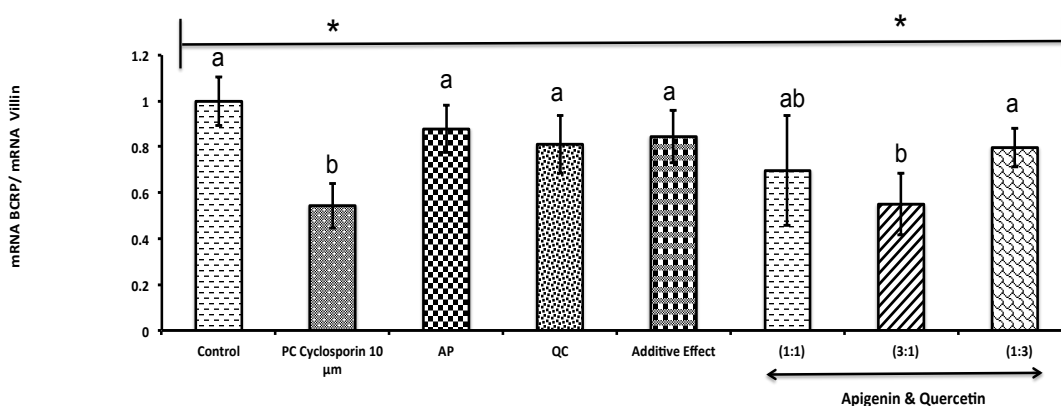
**B**



**Figure 58:** Effect of apigenin and quercetin mixtures on multidrug resistant associated protein 3 (MRP3) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with apigenin and quercetin (0.01  $\mu$ M) combinations; and analyzed by western blot as ratio to  $\beta$ -actin protein (**A**). Western blot analysis of MRP3 (**B**). Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

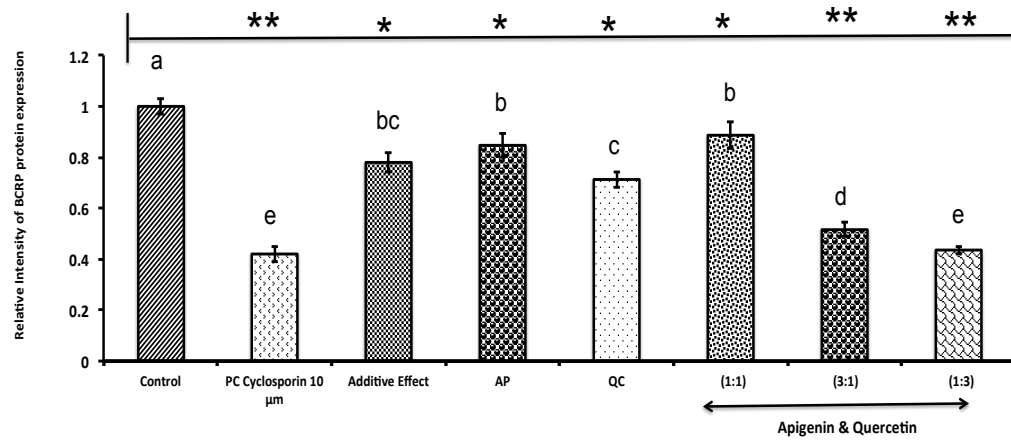


**Figure 59:** Effect of apigenin and quercetin mixtures on breast cancer resistance protein (BCRP) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin combinations at 0.05-1 µM; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

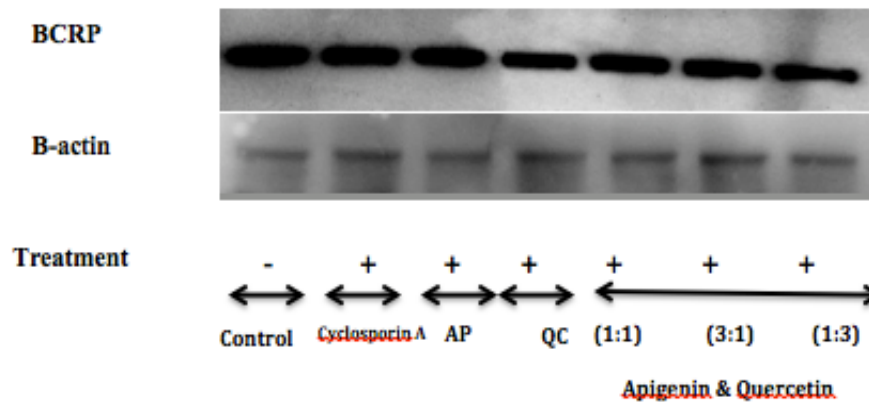


**Figure 60:** Effect of apigenin and quercetin mixtures on breast cancer resistance protein (BCRP) gene expression in Caco-2 monolayers. Cells were pre-treated for 6 h with apigenin and quercetin (0.05 µM) combinations; and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

**A**



**B**



**Figure 61:** Effect of apigenin and quercetin mixtures on breast cancer resistance protein (BCRP) protein expression in Caco-2 monolayers. Cells were pre-treated for 24 h with apigenin and quercetin (0.05 µM) combinations; and analyzed by western blot as ratio to β-actin protein (A). Western blot analysis of BCRP (B). Values are means ± SD (n = 3). (\*) indicate significance compared to control (non-treated cells) at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporine A-treated cells).

## Conclusions

The White sorghum-white cowpea combinations were able to synergistically modify the active efflux transporters in Caco-2 cell monolayers by inhibiting the expression of apically located ABC transporters including BCRP, MRP2, MRP3 and MDR1 at both mRNA and protein levels. The combination of apigenin and quercetin significantly enhanced their operation in modulating efflux transporters by reducing the same biomarker expression. The synergistic inhibitory role against transporters by apigenin-quercetin mixtures was adequate to explain the synergistic interaction execution of sorghum-cowpea mixtures against ABC transporters. Thus, interactive effect in transporters modulation revealed in combined flavonoids could be a mechanism for sorghum-cowpea enhanced action against inflammation.

The magnitude of synergy was strongly dependent on the relative proportions in both sorghum-cowpea and apigenin-quercetin mixtures, which suggest different modes of action for the flavones and flavonols. Overall, structural differences in major types of flavonoids present in sorghum and cowpea resulted in enhanced action in their capacity to mitigate expression of efflux transporters genes and proteins in Caco-2 cell monolayers. The co-administration of specific flavonoids may lead to an effective way to improve flavonoid bioavailability, and allow flavonoids to exert better bioactivity in humans. These results suggest that the combinations of white sorghum and white cowpea could be considered a viable source of dietary polyphenols capable of inhibit the detrimental effects of chronic disease.



## **CHAPTER VI**

### **CONCLUSION AND FUTURE WORKS**

Overall, structural differences in major types of flavonoids present in sorghum and cowpea resulted in enhanced (2-10 fold) action in their capacity to modulate the ROS generation and markers linked to colon inflammation. Consistent synergistic effect in down-regulating efflux transporter gene and protein expression in Caco-2 cell monolayers was observed. This suggests the sorghum-cowpea flavonoids combinations may enhance anti-inflammatory effect by cooperatively inhibiting the membrane transporter proteins, and thus effectively becomes more bioavailable.

These results suggest that consuming food together (in this case cereal and legume) is likely to increase health outcomes beyond what can be observed by measuring individual components. Our study is especially important given that sorghum and cowpea are usually produced and consumed together, especially by populations likely to benefit a great deal from protective effect of the bioactive constituents of these commodities. Consuming sorghum and cowpea contribute to the health of vulnerable populations in low-income regions by reducing protein malnutrition. The enhanced anti-inflammatory capacity of sorghum-cowpea combinations is likely to further help protect the population against chronic disease by improving immune function. Furthermore, populations in the developed world are constantly looking for novel foods that can help prevent the chronic diseases that affect these regions. Thus sorghum and cowpea present an opportunity to develop novel consumer foods that provide health-promoting properties.

Further studies are needed to better understand the potential consequences of sorghum-cowpea flavonoid combinations on chronic disease prevention. Follow up studies could focus on:

- 1- Identifying molecular mechanisms for synergistic anti-inflammatory effect of sorghum-cowpea flavonoid mixtures *in vivo*.
- 2- Determining effect of co-administration of sorghum-cowpea flavonoids on phase II enzymes and metabolic pathway.
- 3- Investigating effect of co-administration of sorghum-cowpea flavonoids on the efflux of flavonoids and their phase II metabolites.
- 4- Understanding the interactions among different flavonoids in crude sorghum-cowpea, to identify mixtures with the best potential to enhance health benefits of foods.

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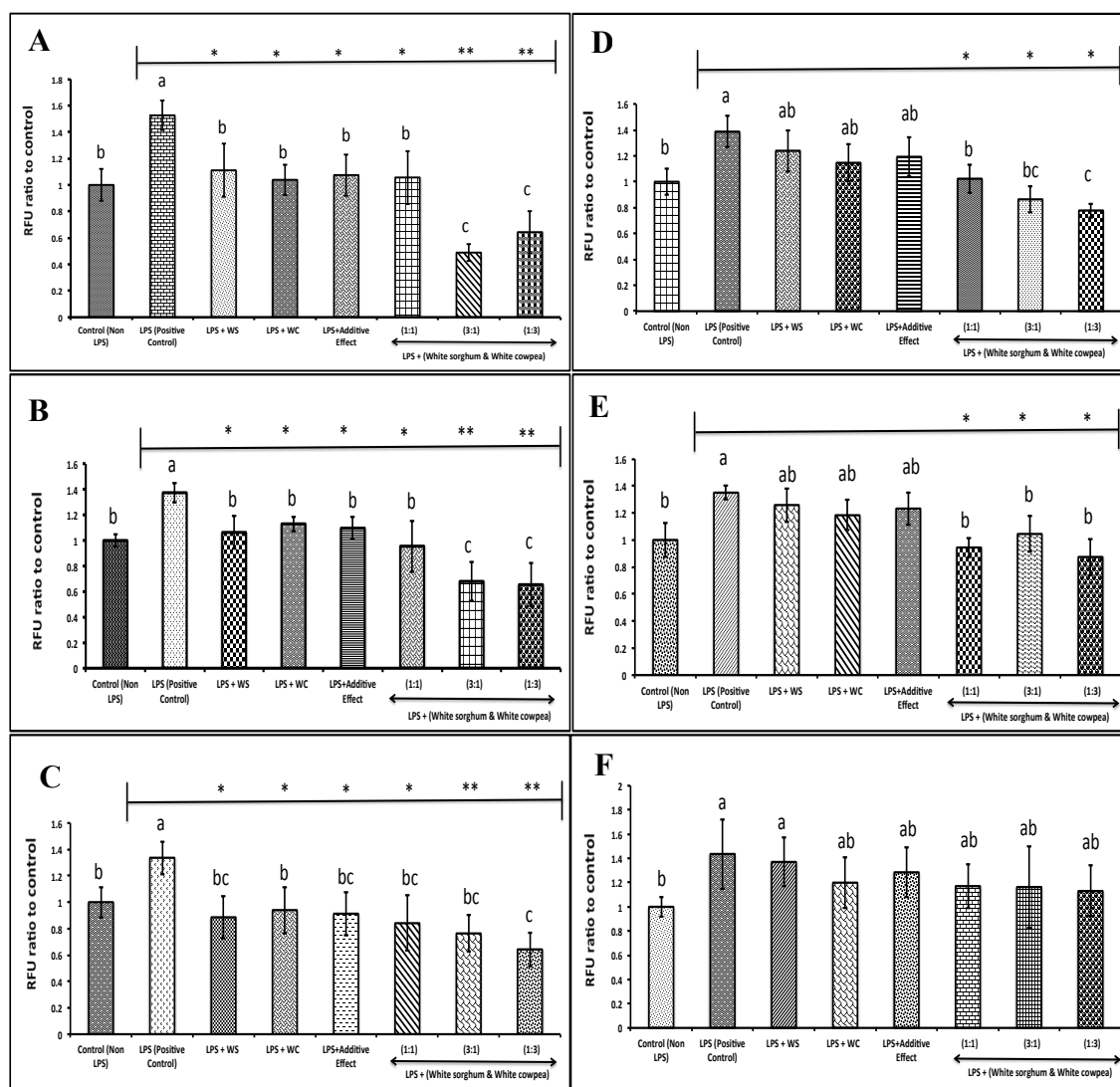
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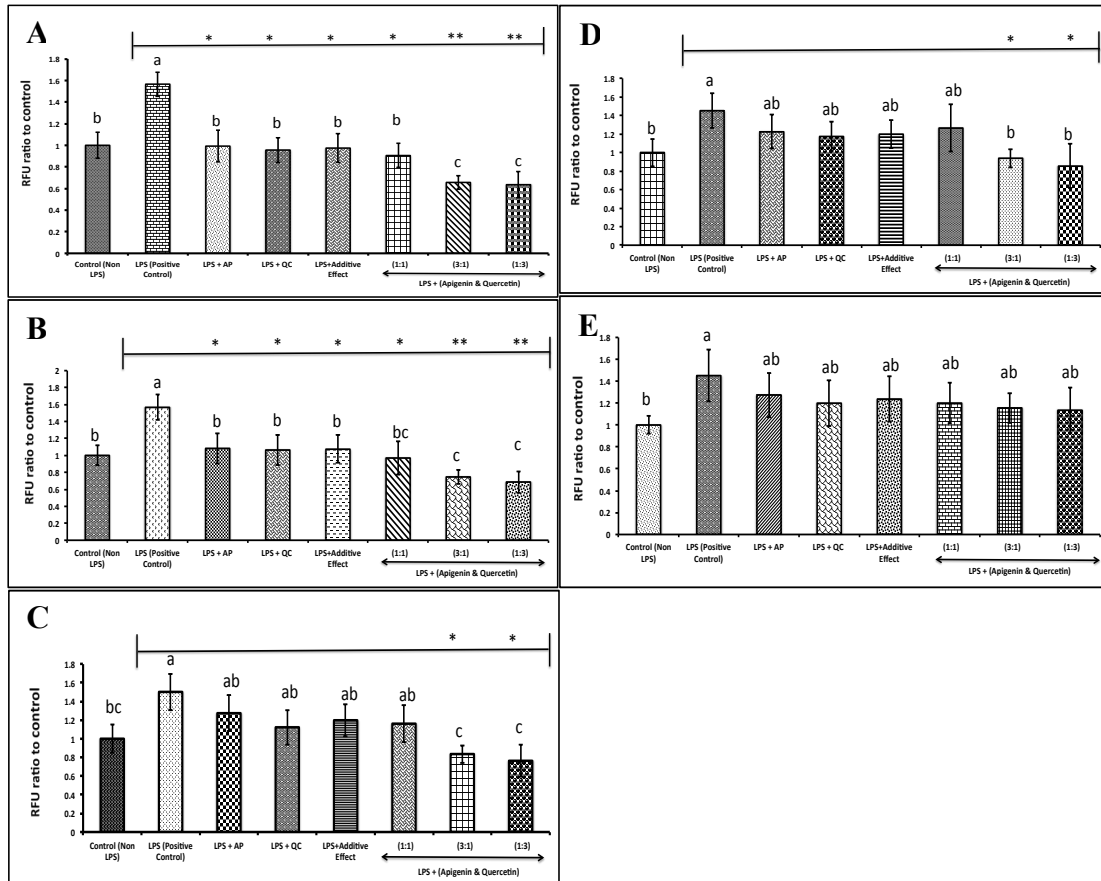
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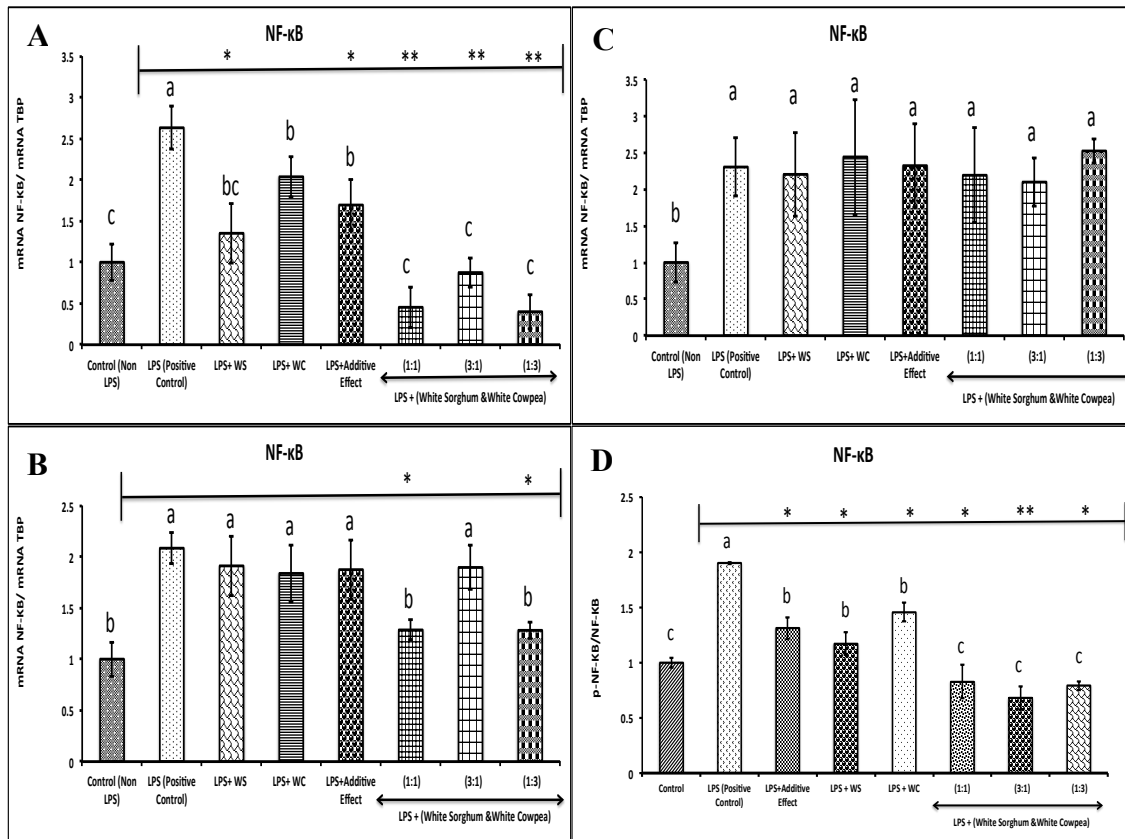
## APPENDIX



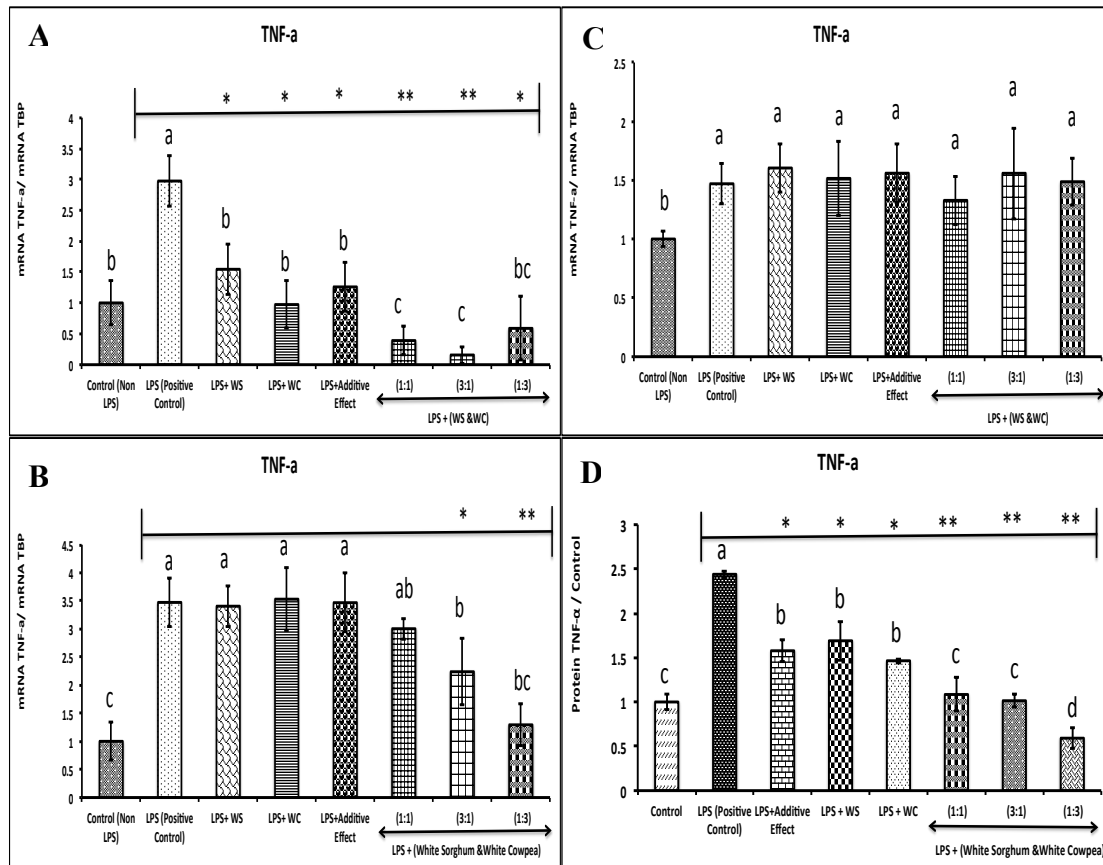
**Figure A-1:** Effect of sorghum-cowpea extracts on generation of ROS after LPS-induced oxidative stress in non-cancer CCD-18Co cells. Cells were pretreated with extracts **(A):** (100 µg/g) **(B):** (10 µg/g) **(C):** (1 µg/g) **(D):** (0.1 µg/g) **(E):** (0.05 µg/g) and **(F):** (0.01 µg/g) from sorghum-cowpea mixtures at different ratios (1:3-3:1) for 24 h. Values are means  $\pm$  SD,  $n = 3$ ; (\*) indicate significant difference at  $p < 0.05$  compared to positive control. Different letters indicate significant difference at  $p < 0.05$  compared to non-LPS treated control.



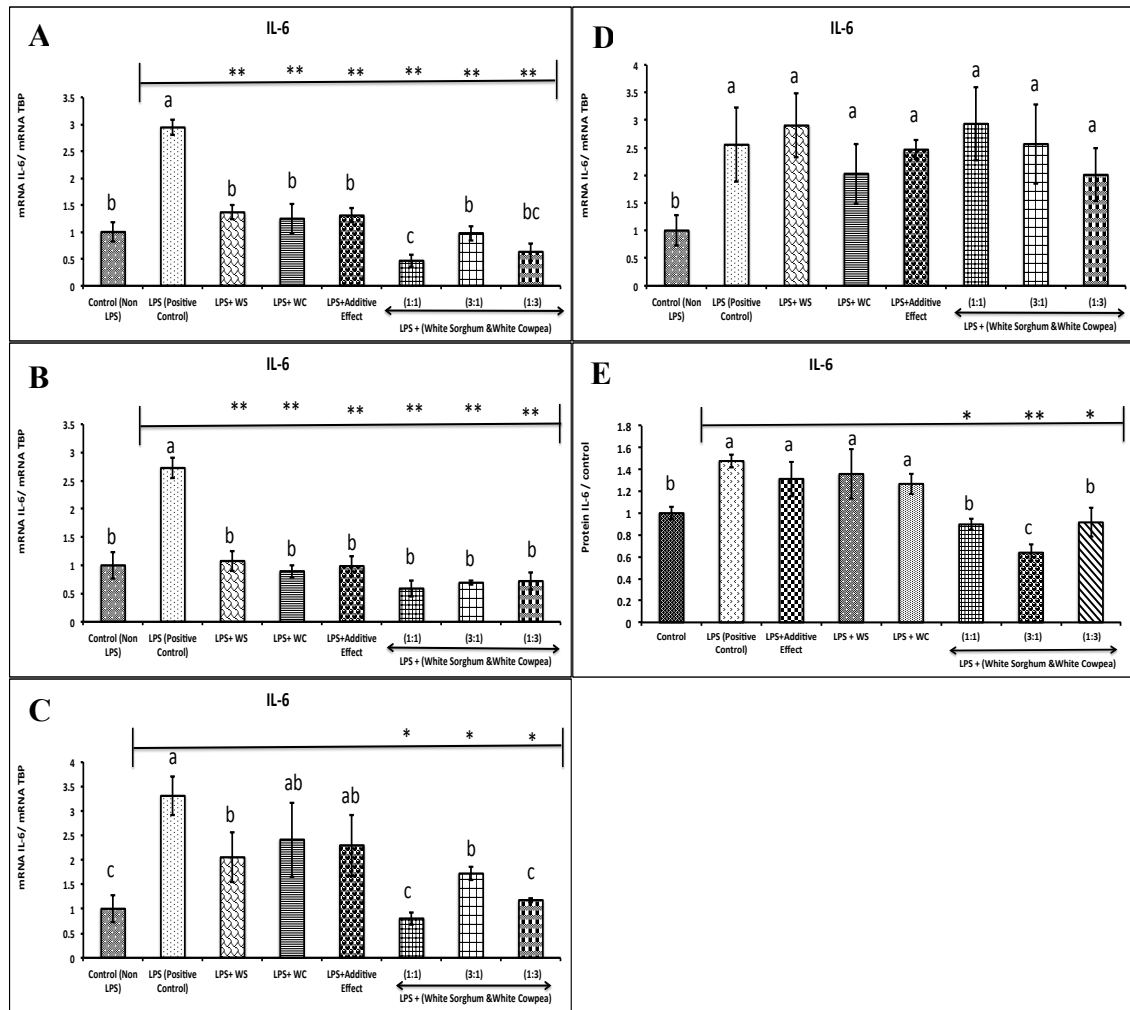
**Figure A-2:** Effect of apigenin-quercetin extracts on generation of ROS after LPS-induced oxidative stress in non-cancer CCD-18Co cells. Cells were pretreated with apigenin-quercetin mixtures extracts **A**: (1.0  $\mu M$ ), **B**: (0.1  $\mu M$ ), **C**: (0.05  $\mu M$ ), **D**: (0.01  $\mu M$ ) and **E**: (0.005  $\mu M$ ) at different ratios (1:3-3:1) for 24 h. Values are  $\pm$  SD,  $n = 3$ ; (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to non-LPS treated control.



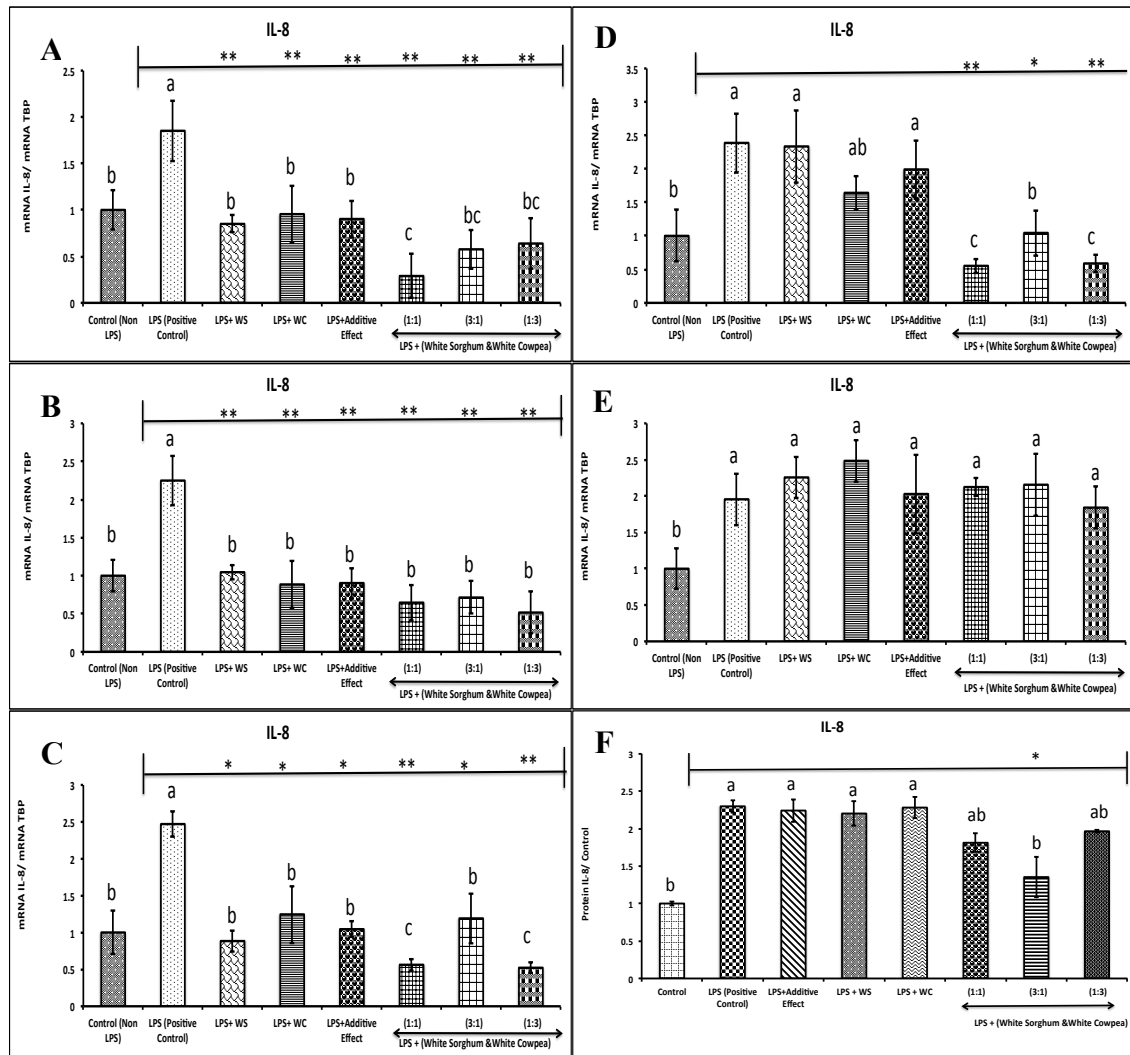
**Figure A-3:** Effect of extracts of sorghum and cowpea mixtures on nuclear factor-  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at **(A):** 1.0  $\mu\text{g/mL}$ ; **(B):** 0.1  $\mu\text{g/mL}$ ; **(C):** 0.05  $\mu\text{g/mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. **(D):** CCD-18Co protein expression of NF- $\kappa\text{B}$  and pNF- $\kappa\text{B}$ . Cells were treated with different (0.1  $\mu\text{g/mL}$ ) sorghum and cowpea combinations for 24 h, then challenged with LPS for 24 h. Protein expression was analyzed by Western Blot. Anti-inflammatory effect of sorghum and cowpea combinations at (0.1  $\mu\text{g/mL}$ ), based on the ratio of quantified p-NF- $\kappa\text{B}$ /NF- $\kappa\text{B}$  activation, normalized with untreated control cells. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



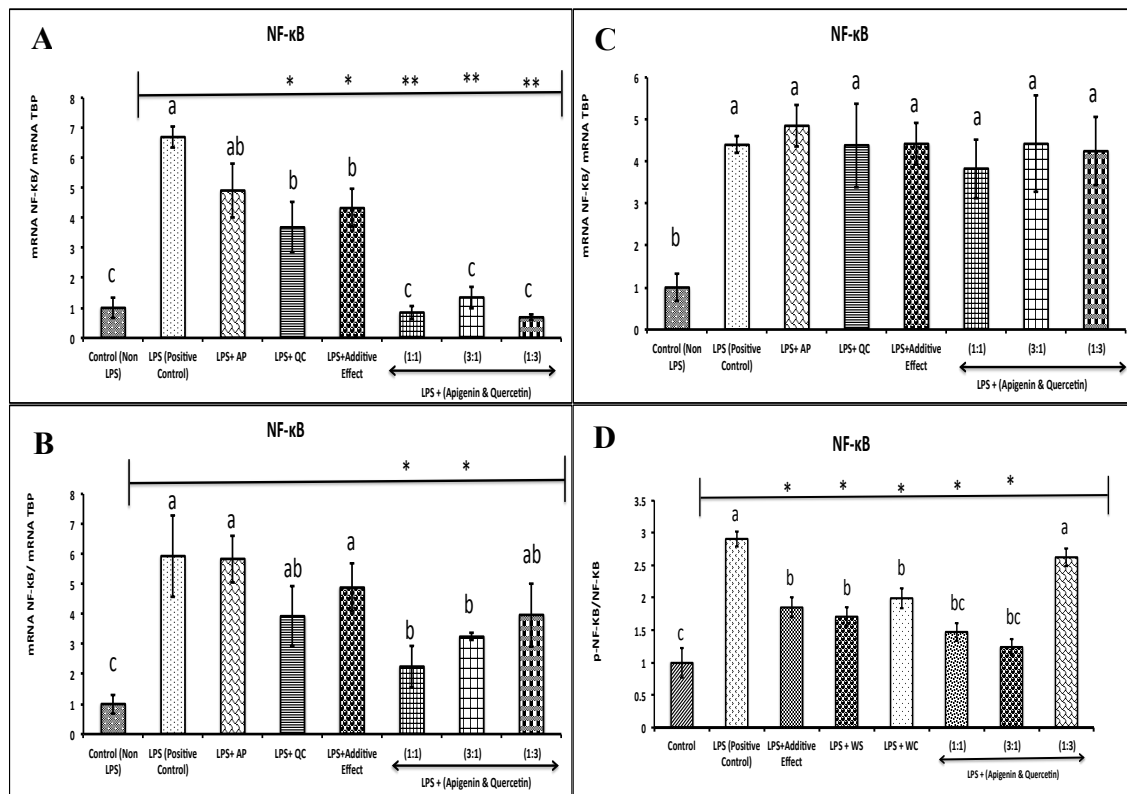
**Figure A-4:** Effect of extracts of sorghum and cowpea mixtures on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at **(A):** 1.0  $\mu$ g/mL; **(B):** 0.1  $\mu$ g/mL; **(C):** 0.05  $\mu$ g/mL, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. **(D):** CCD-18Co protein expression of TNF- $\alpha$ . Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ g/ml) of sorghum and cowpea combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



**Figure A-5:** Effect of extracts of sorghum and cowpea mixtures on interleukin-6 (IL-6) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at **(A):** 1.0 µg/mL; **(B):** 0.1 µg/mL; **(C):** 0.05 µg/mL; **(D):** 0.01 µg/mL, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. **(E):** CCD-18Co protein expression of IL-6. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.05 µg/ml) of sorghum and cowpea combinations, and challenged with LPS (10 µg/mL) for 24 h were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

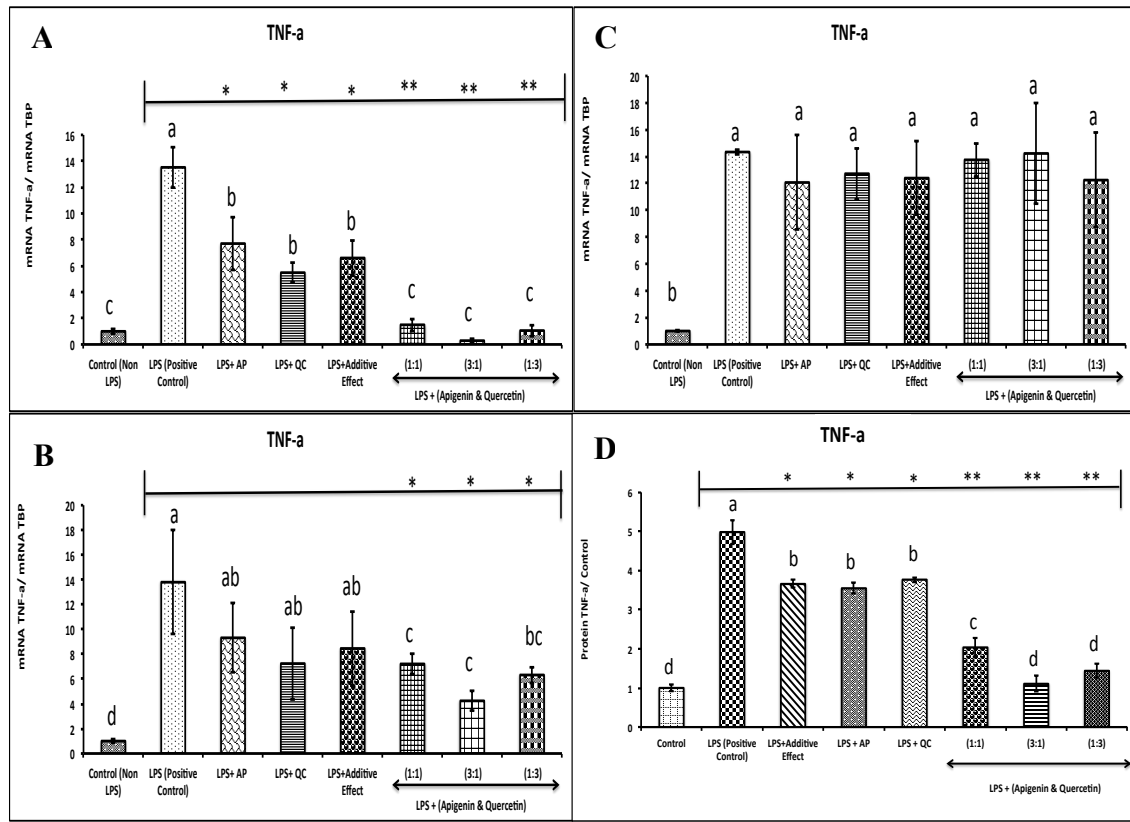


**Figure A-6:** Effect of extracts of sorghum and cowpea mixtures on interleukin-8 (IL-8) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of sorghum and cowpea combinations at (A): 1.0  $\mu\text{g/mL}$ ; (B): 0.1  $\mu\text{g/mL}$ ; (C): 0.05  $\mu\text{g/mL}$ ; (D): 0.01  $\mu\text{g/mL}$ ; (E): 0.005  $\mu\text{g/mL}$ , then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. (F): CCD-18Co protein expression of IL-8. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.01  $\mu\text{g/mL}$ ) of sorghum and cowpea combinations, and challenged with LPS (10  $\mu\text{g/mL}$ ) for 24 h were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

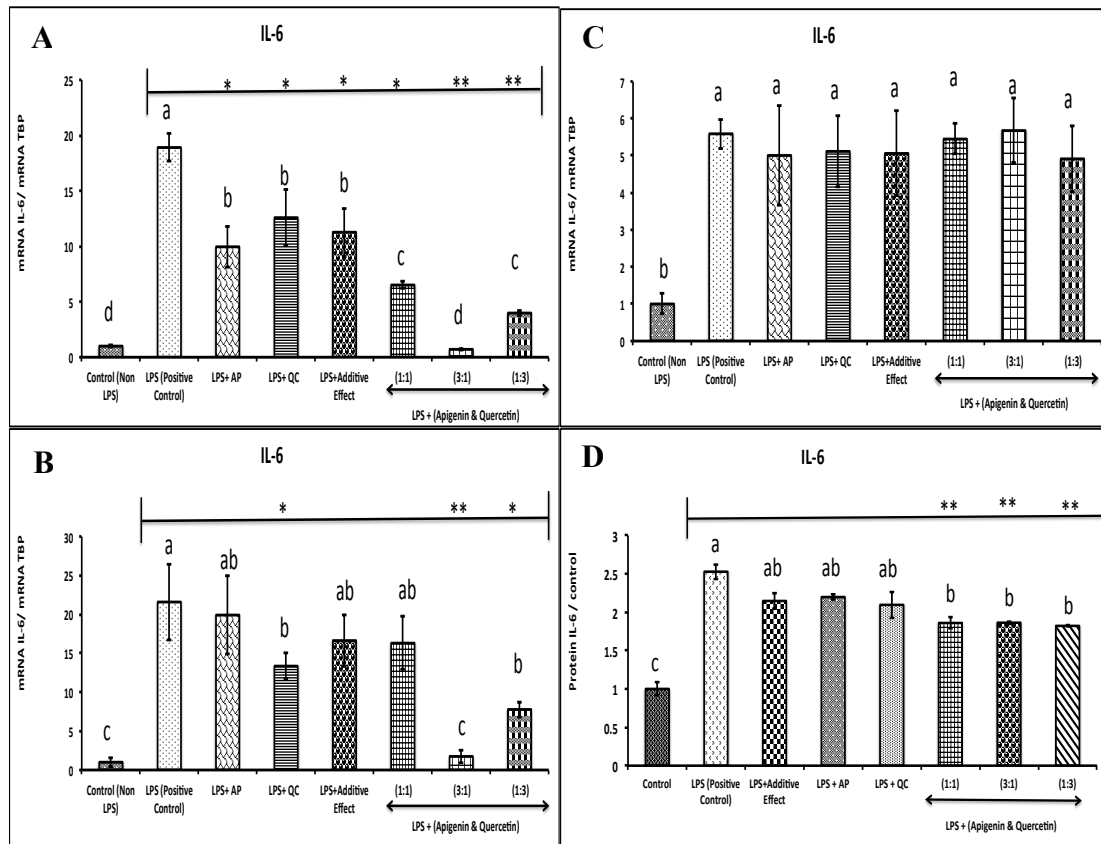


**Figure A-7:** Effect of extracts of apigenin and quercetin mixtures on nuclear factor-  $\kappa$ B (NF- $\kappa$ B) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at **(A):** 1.0  $\mu$ M; **(B):** 0.1  $\mu$ M; **(C):** 0.05  $\mu$ M then challenged with LPS (10  $\mu$ g/mL) for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. **(D):** CCD-18Co protein expression of NF- $\kappa$ B and pNF- $\kappa$ B. Cells were treated with different (0.1  $\mu$ M) apigenin and quercetin combinations for 24 h, then challenged with LPS for 24 h. Protein expression was analyzed by western Blot. Anti-inflammatory effect of apigenin and quercetin combinations at (0.1  $\mu$ M), based on the ratio of quantified p-NF- $\kappa$ B/NF- $\kappa$ B activation, normalized with untreated control cells. Values are means  $\pm$  SD (n = 3). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.

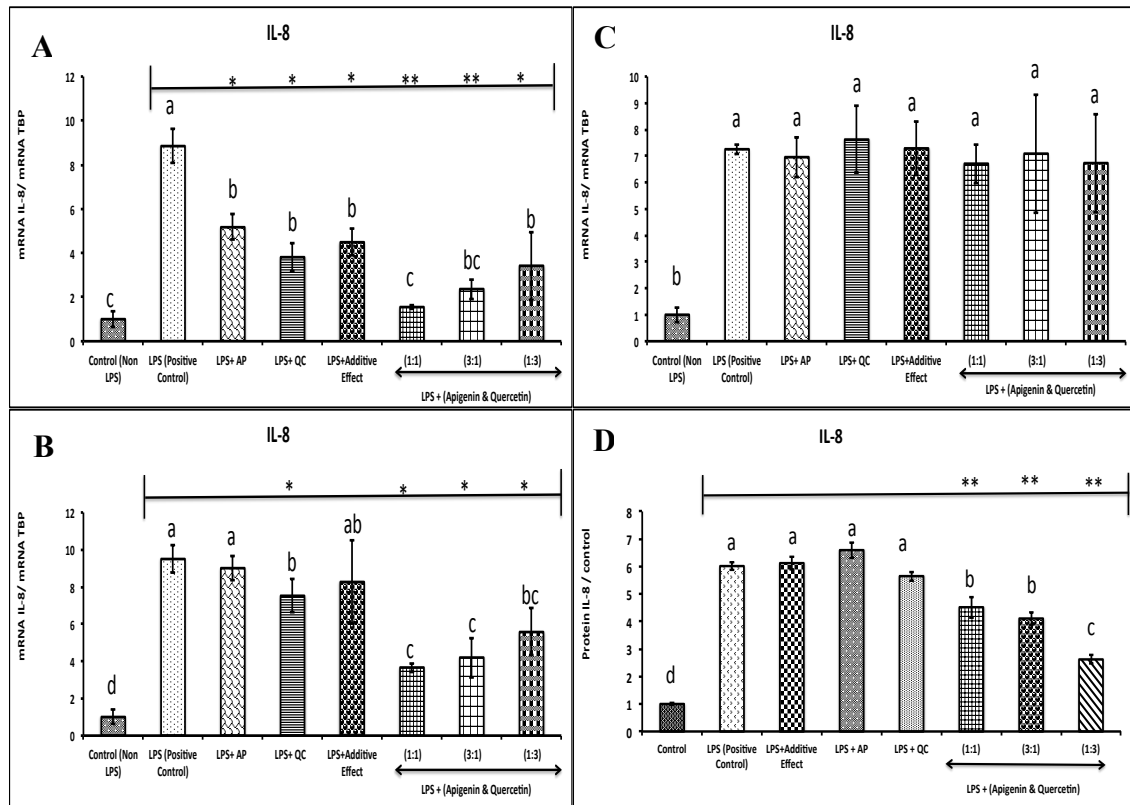




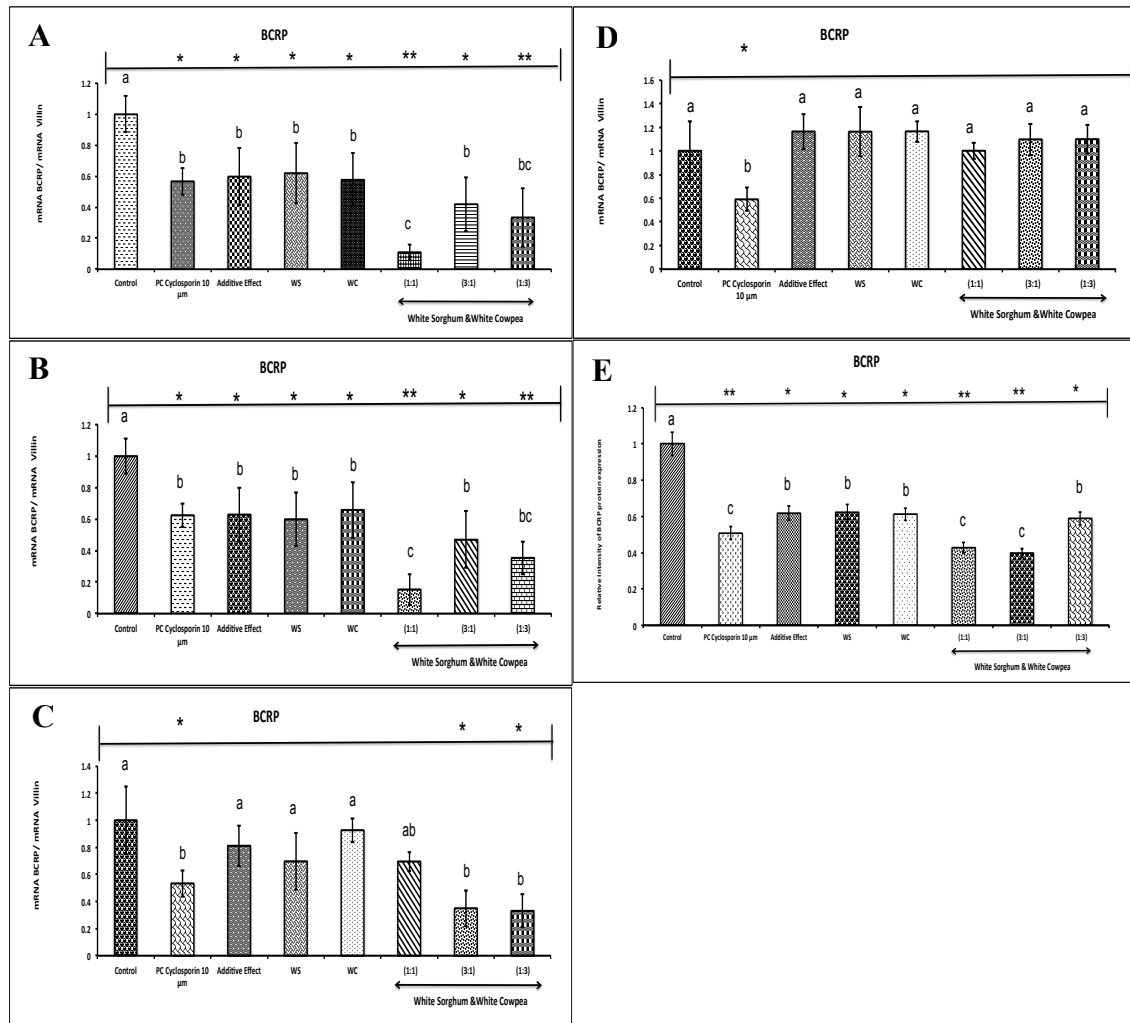
**Figure A-8:** Effect of extracts of apigenin and quercetin mixtures on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at (A): 1.0  $\mu$ M; (B): 0.1  $\mu$ M; (C): 0.05  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. (D): CCD-18Co protein expression of TNF- $\alpha$ . Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ M) of apigenin and quercetin combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



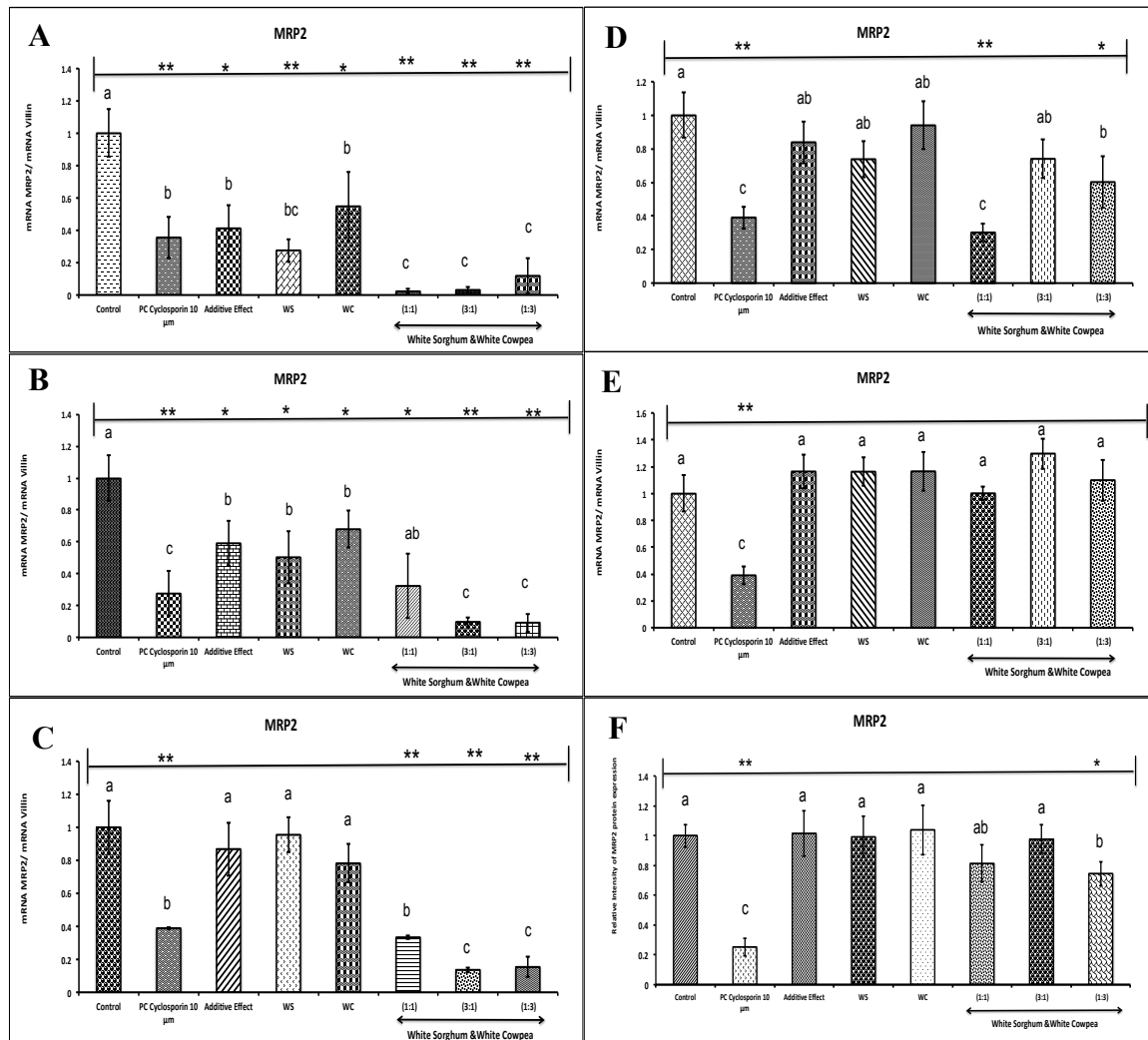
**Figure A-9:** Effect of extracts of apigenin and quercetin mixtures on interleukin-6 (IL-6) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at **(A):** 1.0  $\mu$ M; **(B):** 0.1  $\mu$ M; **(C):** 0.05  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. **(D):** CCD-18Co protein expression of IL-6. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ M) of apigenin and quercetin combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



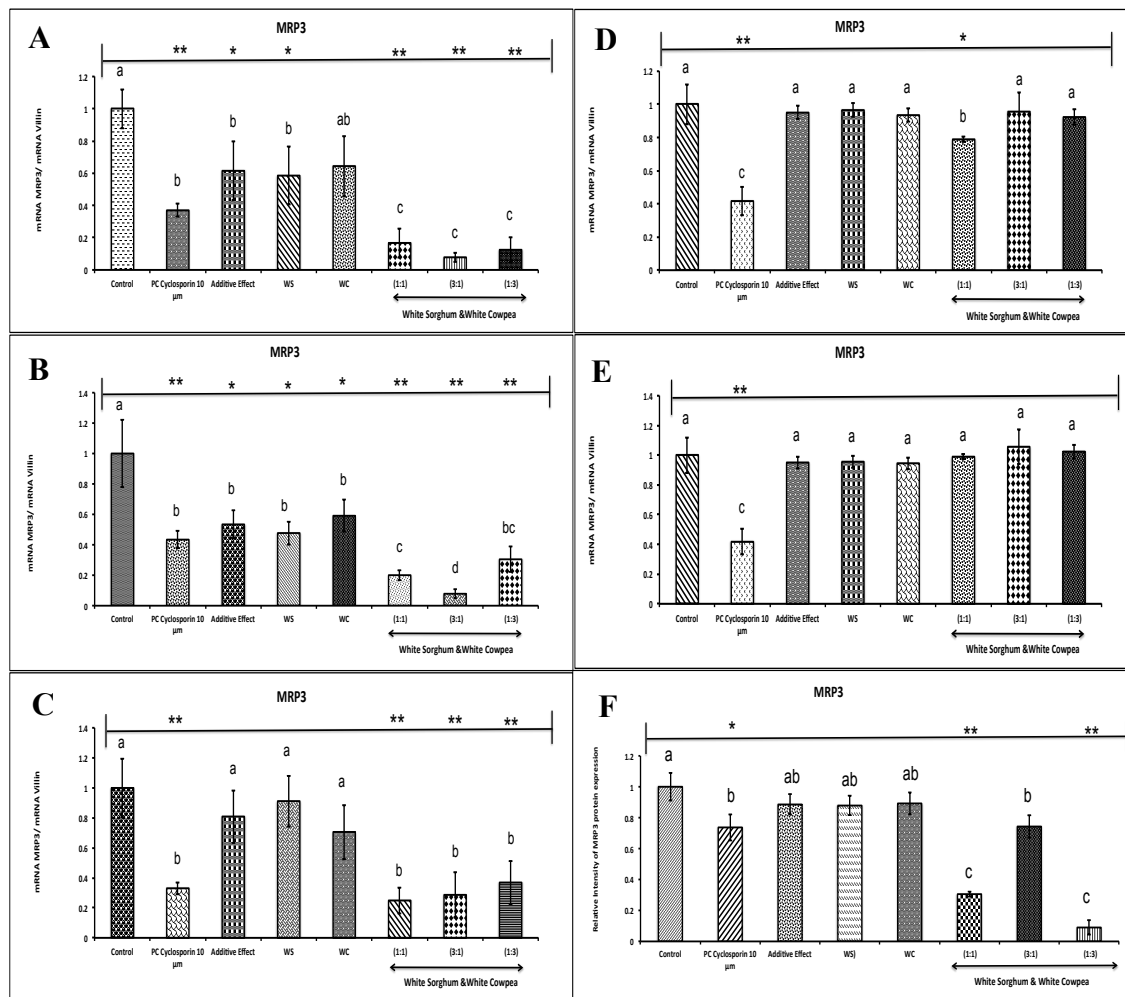
**Figure A-10:** Effect of extracts of apigenin and quercetin mixtures on interleukin-8 (IL-8) gene and protein expression in CCD-18Co cells. Cells were pre-treated for 9 h with extracts of apigenin and quercetin combinations at **(A):** 1.0  $\mu$ M; **(B):** 0.1  $\mu$ M; **(C):** 0.05  $\mu$ M, then challenged with LPS for 9 h; and analyzed by real time qRT-PCR as ratio to TBP mRNA. **(D):** CCD-18Co protein expression of IL-8. Cell culture supernatants from cells pretreated for 24 h with solvent (DMSO) or extracts (0.1  $\mu$ M) of apigenin and quercetin combinations, and challenged with LPS (10  $\mu$ g/mL) for 24 h were analyzed by multiplex bead analysis. Values were normalized to protein concentrations relative to untreated control cells. Values are means  $\pm$  SE ( $n = 3$ ). (\*) indicate significance compared to positive control at  $p < 0.05$ ; (\*\*) at  $p < 0.01$ . Different letters indicate significant difference at  $p < 0.05$  compared to negative control.



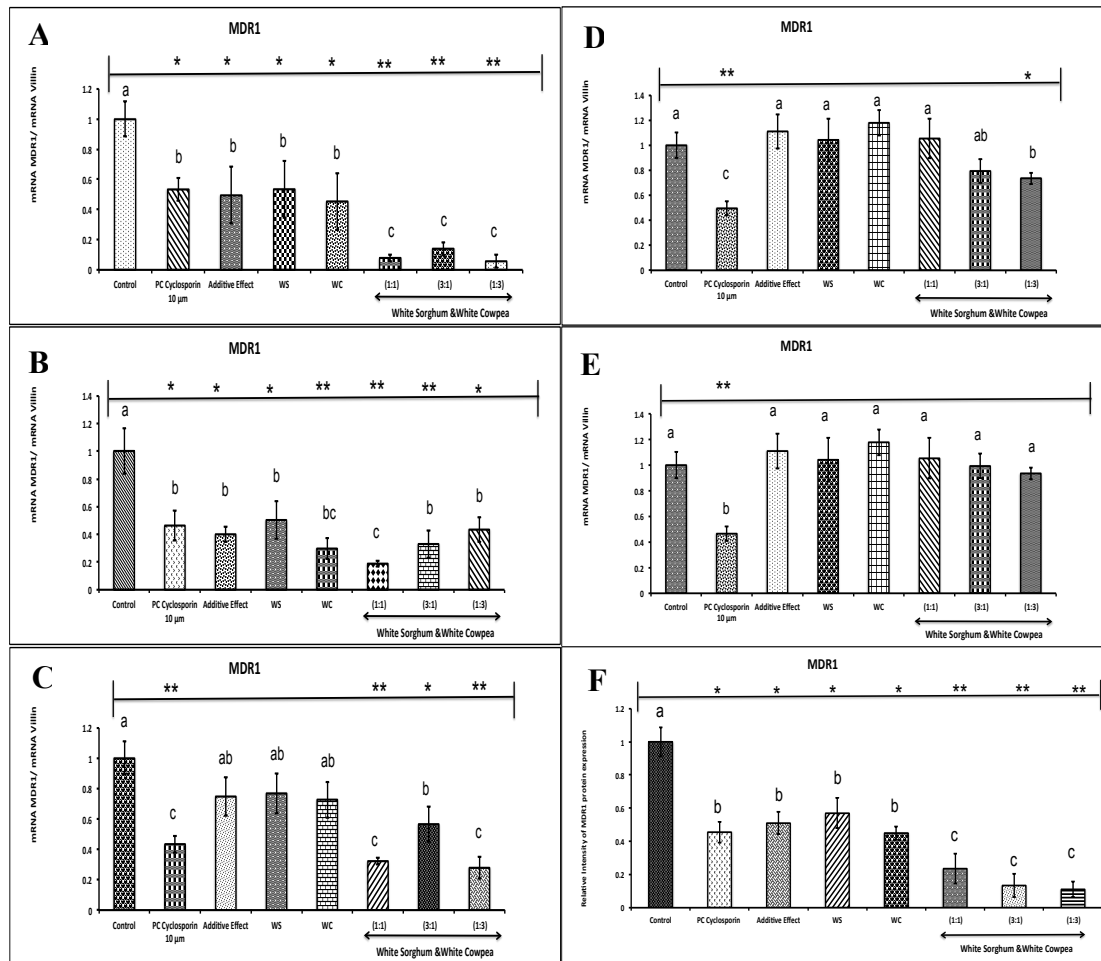
**Figure A-11:** Effect of extracts of sorghum and cowpea mixtures on breast cancer resistance protein (BCRP) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of sorghum and cowpea combinations in dose dependent concentration from (A):(1  $\mu\text{g/mL}$ ), (B):(0.1  $\mu\text{g/mL}$ ), (C):(0.05  $\mu\text{g/mL}$ ) to (D):(0.01  $\mu\text{g/mL}$ ) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (E): Caco-2 monolayers protein expression of BCRP. Cells were pre-treated for 24 h with extracts (0.05  $\mu\text{g/mL}$ ) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).



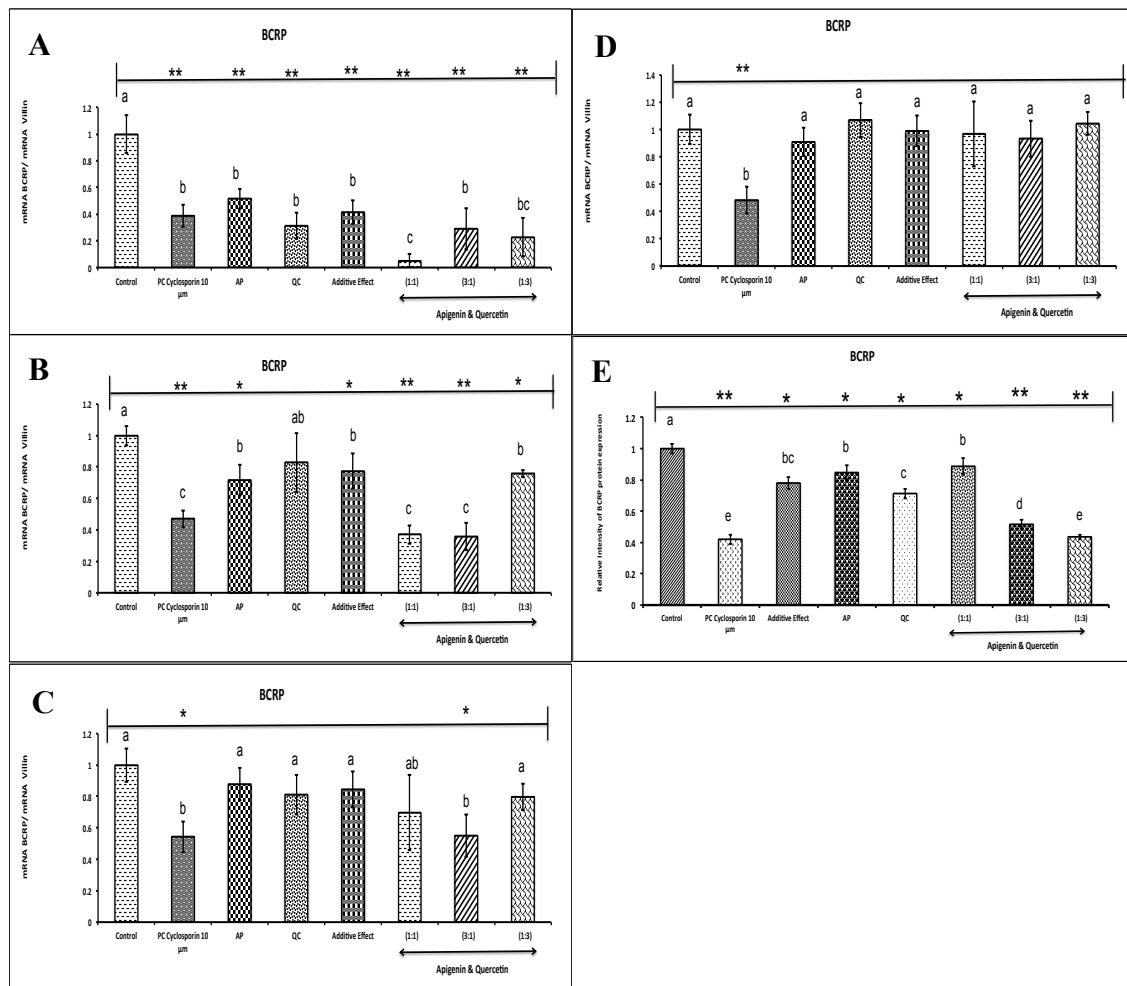
**Figure A-12:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 2 (MRP2) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of sorghum and cowpea combinations in dose dependent concentration from **(A):**(1  $\mu$ g/mL), **(B):**(0.1  $\mu$ g/mL), **(C):**(0.05  $\mu$ g/mL), **(D):**(0.01  $\mu$ g/mL) to **(E):**(0.005  $\mu$ g/mL) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). **(F):** Caco-2 monolayers protein expression of MRP2. Cells were pre-treated for 24 h with extracts (0.05  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).



**Figure A-13:** Effect of extracts of sorghum and cowpea mixtures on multidrug resistant associated protein 3 (MRP3) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of sorghum and cowpea combinations in dose dependent concentration from **(A):**(1  $\mu$ g/mL), **(B):**(0.1  $\mu$ g/mL), **(C):**(0.05  $\mu$ g/mL), **(D):**(0.01  $\mu$ g/mL) to **(E):**(0.005  $\mu$ g/mL) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). **(F):** Caco-2 monolayers protein expression of MRP3. Cells were pre-treated for 24 h with extracts (0.05  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).

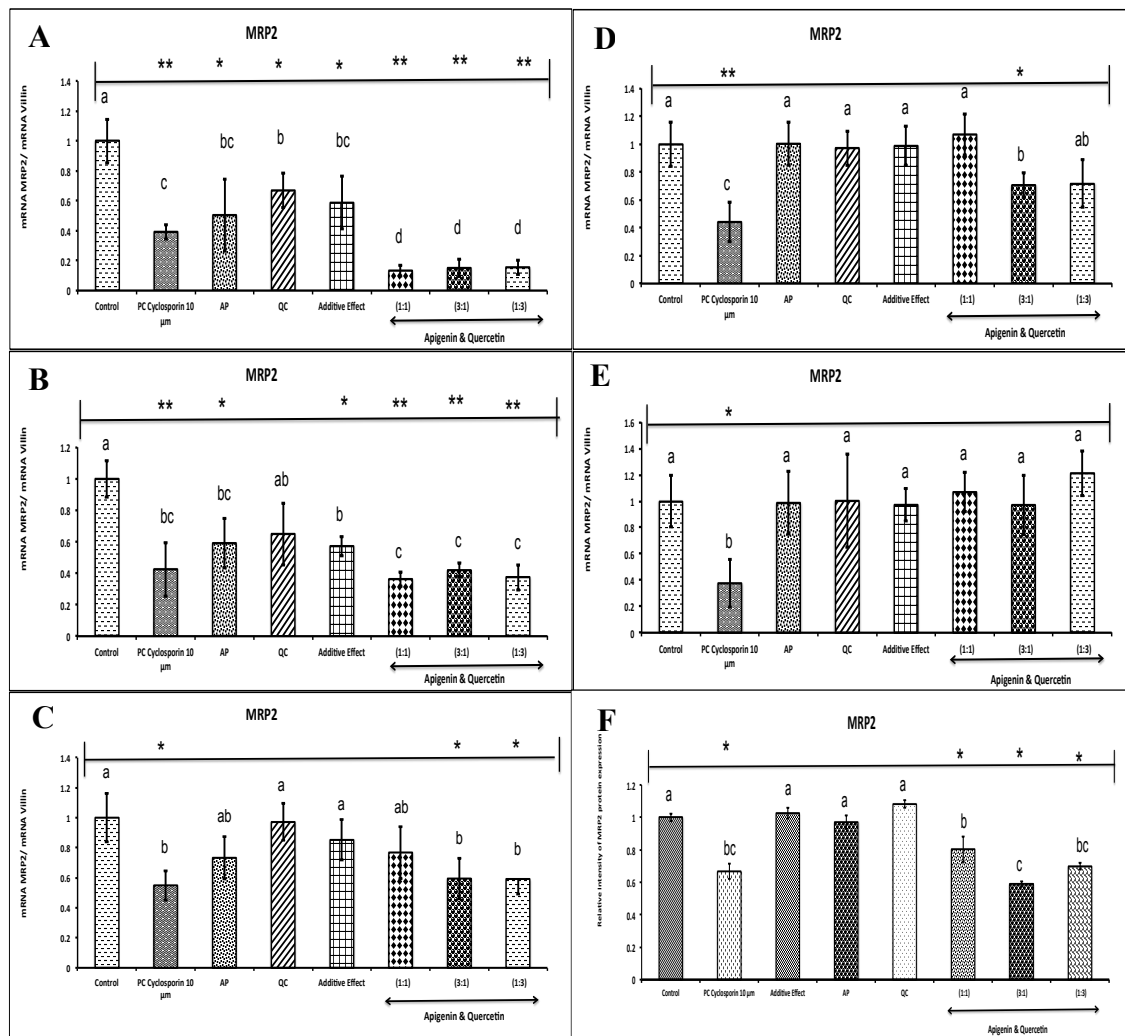


**Figure A-14:** Effect of extracts of sorghum and cowpea mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/P-gp) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of sorghum and cowpea combinations in dose dependent concentration from (A):(1  $\mu$ g/mL), (B):(0.1  $\mu$ g/mL), (C):(0.05  $\mu$ g/mL), (D):(0.01  $\mu$ g/mL) to (E):(0.005  $\mu$ g/mL) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (F): Caco-2 monolayers protein expression of MDR1. Cells were pre-treated for 24 h with extracts (0.05  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).

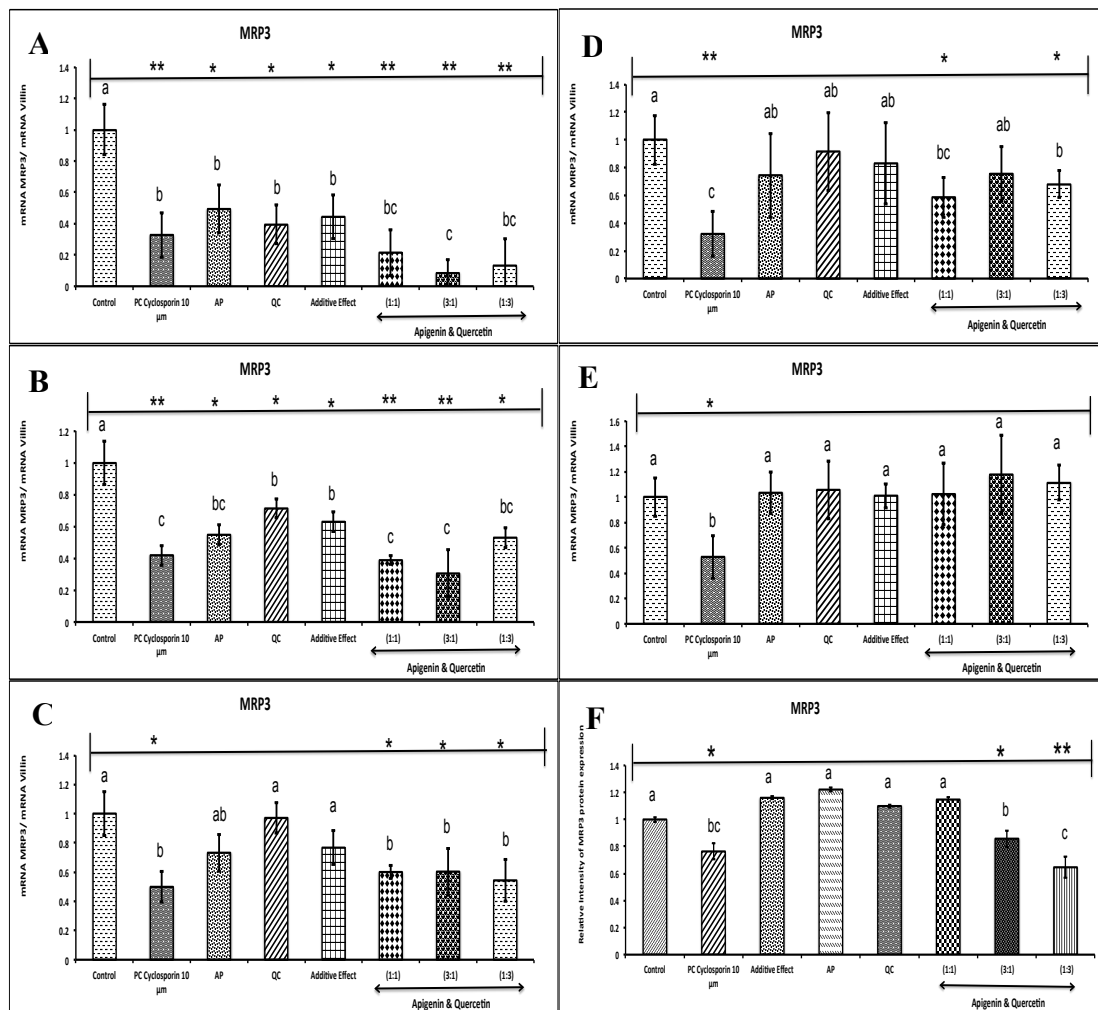


**Figure A-15:** Effect of extracts of apigenin and quercetin mixtures on breast cancer resistance protein (BCRP) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of apigenin and quercetin combinations in dose dependent concentration from **(A):**(1  $\mu$ M), **(B):**(0.1  $\mu$ M), **(C):**(0.05  $\mu$ M) to **(D):**(0.01  $\mu$ M) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). **(E):** Caco-2 monolayers protein expression of BCRP. Cells were pre-treated for 24 h with extracts (0.05  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).

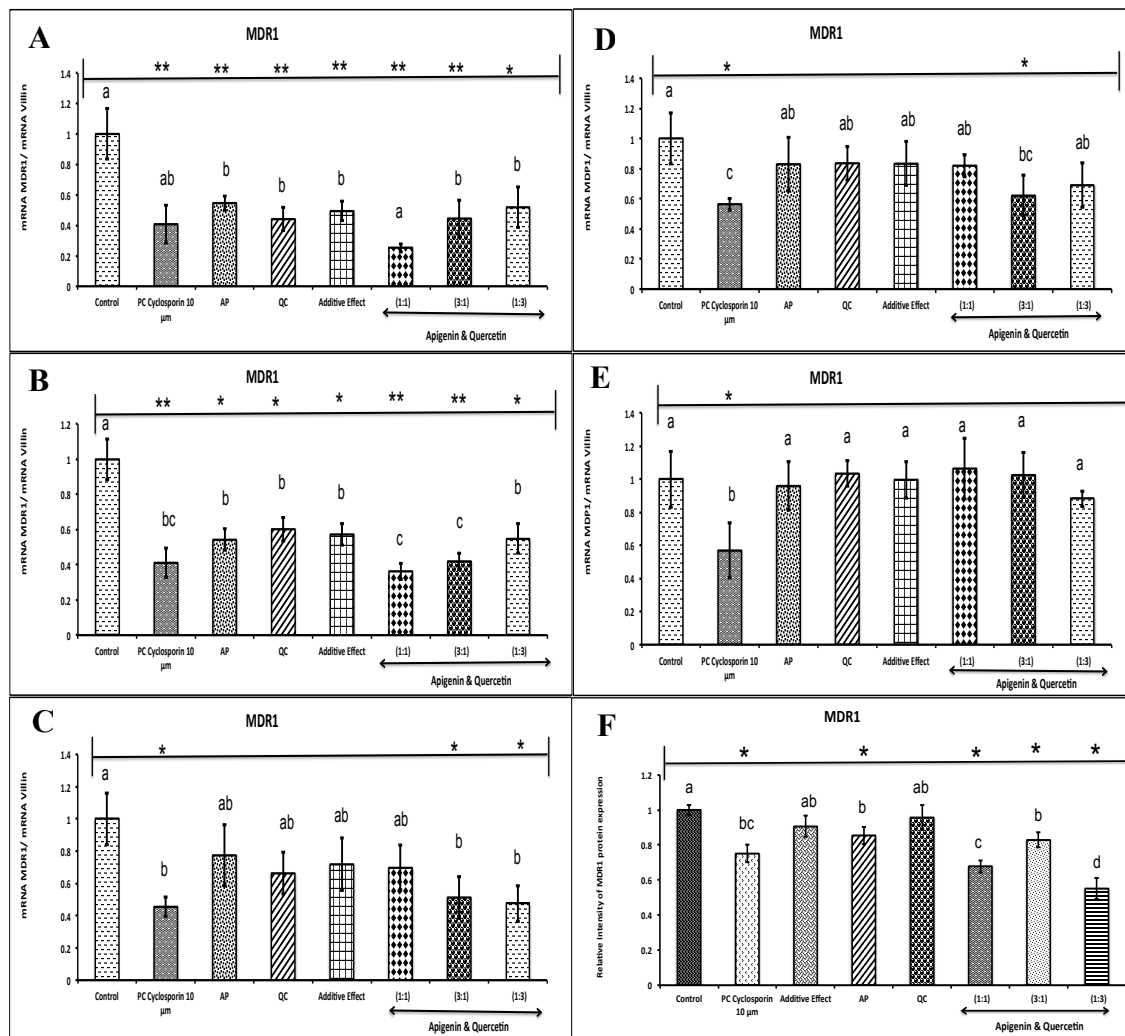




**Figure A-16:** Effect of extracts of apigenin and quercetin mixtures on multidrug resistant associated protein 2 (MRP2) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of apigenin and quercetin combinations in dose dependent concentration from (A):(1 μM), (B):(0.1 μM), (C):(0.05 μM), (D):(0.01 μM) to (E):(0.005 μM) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means ± SE (n = 3). (F): Caco-2 monolayers protein expression of MRP2. Cells were pre-treated for 24 h with extracts (0.05 μg/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to β-actin protein. (\*) indicate significant difference at p < 0.05; (\*\*) at p < 0.01 compared to control (non-treated cells). Different letters indicate significant difference at p < 0.05 compared to positive control (cyclosporin A treated cells).



**Figure A-17:** Effect of extracts of apigenin and quercetin mixtures on multidrug resistant associated protein 3 (MRP3) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of apigenin and quercetin combinations in dose dependent concentration from **(A):**(1  $\mu$ M), **(B):**(0.1  $\mu$ M), **(C):**(0.05  $\mu$ M), **(D):**(0.01  $\mu$ M) to **(E):**(0.005  $\mu$ M) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). **(F):** Caco-2 monolayers protein expression of MRP3. Cells were pre-treated for 24 h with extracts (0.05  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).



**Figure A-18:** Effect of extracts of apigenin and quercetin mixtures on multi-drug resistant protein1/P-glycoprotein (MDR1/P-gp) gene and protein expression in Caco-2 monolayers. Cells were pre-treated for 6 h with extracts of apigenin and quercetin combinations in dose dependent concentration from (A):(1  $\mu$ M), (B):(0.1  $\mu$ M), (C):(0.05  $\mu$ M), (D):(0.01  $\mu$ M) to (E):(0.005  $\mu$ M) and analyzed by real time qRT-PCR as ratio to Villin mRNA. Values are means  $\pm$  SE (n = 3). (F): Caco-2 monolayers protein expression of MDR1. Cells were pre-treated for 24 h with extracts (0.05  $\mu$ g/mL) of sorghum and cowpea combinations; and analyzed by western blot analysis as ratio to  $\beta$ -actin protein. (\*) indicate significant difference at  $p < 0.05$ ; (\*\*) at  $p < 0.01$  compared to control (non-treated cells). Different letters indicate significant difference at  $p < 0.05$  compared to positive control (cyclosporin A treated cells).